Anti-Gout Agent Allopurinol Exerts Cytotoxicity to Human Hormone-Refractory Prostate Cancer Cells in Combination with Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand

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Abstract

Allopurinol has been used for the treatment of gout and conditions associated with hyperuricemia for several decades. We explored the potential of allopurinol on cancer treatment. Allopurinol did not expose cytotoxicity as a single treatment in human hormone refractory prostate cancer cell lines, PC-3 and DU145. However, allopurinol drastically induced apoptosis of PC-3 and DU145 in combination with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), which is a promising candidate for anticancer agent but its efficacy is limited by the existence of resistant cancer cells. We examined the underlying mechanism by which allopurinol overcomes the resistance of prostate cancer cells to TRAIL. Allopurinol up-regulated the expression of a proapoptotic TRAIL receptor, death receptor 5 (DR5). Allopurinol increased DR5 protein, mRNA, and promoter activity. Using DR5 small interfering RNA (siRNA), we showed that allopurinol-mediated DR5 up-regulation contributed to the enhancement of TRAIL effect by allopurinol. Furthermore, we examined the mechanism of allopurinol-mediated DR5 up-regulation. DR5 promoter activity induced by allopurinol was diminished by a mutation of a CAAT/enhancer binding protein homologous protein (CHOP)-binding site. In addition, allopurinol also increased CHOP expression, suggesting that allopurinol induced DR5 expression via CHOP. Allopurinol possesses the activity of a xanthine oxidase (XO) inhibitor. We used XO siRNA instead of allopurinol. XO siRNA also up-regulated DR5 and CHOP expression and sensitized the prostate cancer cells to TRAIL-induced apoptosis. Here, we show the novel potential of allopurinol in cancer treatment and indicate that the combination of allopurinol with TRAIL is effective strategy to expand the TRAIL-mediated cancer therapy. (Mol Cancer Res 2008;6(12):1852–60)

Introduction

Prostate cancer is the most common malignancy and the second leading cause of male cancer death in the United States. The American Cancer Society estimated that, during 2006, about 234,460 new cases of prostate cancer would be diagnosed in the United States and 27,350 men would die of metastatic disease (1). Although androgen ablation is effective in treating prostate cancer, most patients become resistant to hormonal manipulation (2, 3); therefore, new treatment strategies are needed for this disease.

Allopurinol has been a cornerstone of the clinical management of gout and conditions associated with hyperuricemia and has been used worldwide since 1966 (4). Allopurinol acts as a xanthine oxidase (XO) inhibitor and recent data indicate that XO also plays an important role in various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases, and chronic heart failure (5-7). Allopurinol has shown a beneficial effect in the treatment of these conditions both in experimental animal models and in human clinical trials (5-7). Thus, allopurinol has many clinical benefits; however, it has not been applied to cancer treatment.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2L) induces apoptosis selectively in cancer cells in vitro and in vivo and has little or no toxicity to normal cells (8-12). Recombinant TRAIL and agonistic TRAIL receptor antibodies are promising for cancer treatment and going on phase I/II clinical studies (13, 14). TRAIL is a cytokine that is closely related to TNF-α and Fas ligand, members of the TNF family (15). Death receptor 5 (DR5; also called TRAIL-R2) is a receptor for TRAIL. TRAIL induces apoptosis by binding to DR5, causing the formation of a death-inducing signaling complex with binding of caspase-8 (16-21). Autoactivated caspase-8 can directly evoke the cleavage of downstream effector caspases (22, 23). However, some tumor types exhibit resistance to TRAIL (24) and it is important to overcome this resistance.
In the present study, we searched a potential of allopurinol in hormone-resistant prostate cancer treatment and found that allopurinol dramatically causes apoptosis of prostate cancer cells in combination with TRAIL.

**Results**

**Allopurinol Exerts Cytotoxicity of Hormone-Resistant Human Prostate Cancer Cells in Combination with TRAIL**

First, we investigated the cytotoxic effect of allopurinol on hormone-resistant human prostate cancer cells, PC-3 and DU145, as a single agent. As shown in Fig. 1A, allopurinol did not have cytotoxic effect in both PC-3 and DU145 cells. Both cells were also resistant to TRAIL-induced apoptosis (Fig. 1B). Interestingly, allopurinol markedly induced cytotoxic effect on PC-3 and DU145 cells when combined with TRAIL. To elucidate that the sub G1 population caused by the combination of allopurinol and TRAIL is caspase-dependent apoptosis, we used caspase inhibitors. The pan-caspase inhibitor zVAD-fmk efficiently blocked the sub G1 induced by combined treatment with allopurinol and TRAIL (Fig. 1C). These results indicate that the cytotoxic effect mediated by
allopurinol and TRAIL stands for caspase-dependent apoptosis. Moreover, caspase-3–like, caspase-8–like, caspase-9–like, and caspase-10–like inhibitors (25) also interrupted the apoptosis induced by combined treatment. To elucidate whether apoptosis induced by allopurinol and TRAIL occurred via a specific interaction between TRAIL and its receptors, we used a recombinant human DR5/Fc chimeric protein, which has a dominant-negative effect by competing with endogenous TRAIL receptors. As shown in Fig. 1D, the allopurinol-mediated enhancement of TRAIL-induced apoptosis was markedly blocked by DR5/Fc chimera, indicating that allopurinol sensitizes prostate cancer cells to TRAIL-induced apoptosis through specific interactions of TRAIL with its receptors.

Allopurinol Increases a TRAIL Receptor, DR5, Expression in Both PC-3 and DU145 Cells

To elucidate how allopurinol sensitizes prostate cancer cells to TRAIL action, we examined cell surface TRAIL receptor expression by flow cytometry. As shown in Fig. 2, allopurinol increased cell surface DR5 expression in both PC-3 and DU145 cells. In contrast, DR4 and decoy receptor 1 (DcR1) remained unchanged in both cells, although DcR2 was slightly increased.

FIGURE 2. Allopurinol increases cell surface DR5 expression in both PC-3 and DU145 cells. A, PC-3. B, DU145. Cells were treated with 0.1% DMSO or 200 μmol/L allopurinol for 24 h. Subsequently, cells were stained with isotype control IgG and monoclonal antibodies were generated against the extracellular domain of TRAIL receptors DR5, DR4, DcR1, and DcR2. Data were analyzed by flow cytometry. Dotted line histogram, DMSO; solid line histogram, allopurinol.
Allopurinol Exerts Cytotoxicity to Cancer Cells

In only PC-3 cells. These results indicate that allopurinol up-regulates DR5 among TRAIL receptors. We carried out Western blotting to investigate the induction of DR5 by allopurinol at a total protein level. Allopurinol increased DR5 protein in both PC-3 and DU145 cells (Fig. 3A). Moreover, DR5 mRNA was also increased by allopurinol treatment (Fig. 3B). To investigate the further mechanism underlying DR5 up-regulation by allopurinol, we next examined the effect of allopurinol on DR5 promoter activity. We carried out a luciferase assay using reporter plasmids containing the DR5 promoter. Allopurinol significantly enhanced DR5 promoter activity in both PC-3 and DU145 cells (Fig. 3C). These results indicate that allopurinol regulates DR5 expression through transcription.

Up-Regulation of DR5 by Allopurinol Contributes to the Enhancement of TRAIL-Induced Apoptosis

Next, we tested whether up-regulation of DR5 expression by allopurinol has an effect on TRAIL-induced apoptosis. The expression of DR5 protein was efficiently reduced by transiently transfected DR5 small interfering RNA (siRNA; Fig. 4A). This reduction of DR5 expression significantly attenuated the apoptotic response to combined treatment with allopurinol and TRAIL (Fig. 4B). These results suggest that the up-regulation of DR5 expression accounts, at least in part, for the synergistic enhancement of TRAIL-induced apoptosis by allopurinol.

Identification of Allopurinol-Responsive Elements in the DR5 Promoter

As shown in Fig. 3C, allopurinol enhanced DR5 promoter activity in both PC-3 and DU145 cells. Using a series of 5′-deletion mutants, we investigated allopurinol-responsive elements on the DR5 promoter. As shown in Fig. 5A, luciferase activity from pDR5−347 as well as pDR5PF (−2.5 kbp) was increased by allopurinol. On the other hand, pDR5−252 showed a lack of response following allopurinol treatment. These results indicate that the major allopurinol response elements are located between −347 and −253 in the DR5 promoter. This region contains a potential CAAT/enhancer binding protein homologous protein (CHOP)-binding site. To determine whether the site is responsible for transactivation of the DR5 promoter by allopurinol, we carried out a luciferase assay with pDR5/mtCHOP containing a point mutation in the CHOP-binding site. The mutation abolished activation of the DR5 promoter by allopurinol (Fig. 5B). These results suggest that CHOP is associated with DR5 up-regulation by allopurinol.

Allopurinol Increases CHOP Protein, mRNA, and Promoter Activity via Endoplasmic Reticulum Stress Element–Independent Pathway

Allopurinol treatment induced CHOP protein in a dose-dependent manner in both PC-3 and DU145 cells (Fig. 6A). Allopurinol also up-regulated CHOP mRNA (Fig. 6B). To elucidate the mechanism of CHOP up-regulation by allopurinol, we carried out a luciferase assay using reporter plasmids containing the CHOP promoter. Allopurinol increased the promoter activity of CHOP3K, a luciferase reporter plasmid containing a ∼3 kbp fragment of the CHOP promoter region (Fig. 7A). Previous reports have shown that endoplasmic reticulum stress element (ERSE) on the CHOP gene promoter is activated by ER stress triggered by tunicamycin (26). Therefore, to determine whether transactivation of CHOP promoter by allopurinol is caused by ER stress, we carried out a luciferase assay with pCHOP/mtERSE that was developed and described previously (27). The mutation of ERSE abolished the activation of the CHOP promoter by tunicamycin, although
Xanthine Oxidase–Specific siRNA Up-Regulates DR5 Expression and Enhances TRAIL-Induced Apoptosis in PC-3 Cells

To examine the involvement of XO inhibition in the allopurinol effects, we investigated the effect of XO-specific siRNA on DR5 expression. As shown in Fig. 8A, transfection of XO siRNA suppressed XO expression compared with cells transfected with control siRNA. Figure 8A shows the effect of silencing XO on DR5 and CHOP protein expression. XO siRNA up-regulated DR5 and CHOP expression in PC-3 cells. Moreover, combined treatment with XO siRNA and TRAIL markedly induced apoptosis (Fig. 8B). These results indicate that inhibition of XO plays an essential role in the up-regulation of DR5 through CHOP and in enhancing TRAIL-induced apoptosis by allopurinol.

Discussion

To date, anticancer activity of allopurinol has not been reported. In this report, we show for the first time that allopurinol drastically induces apoptosis in human hormone-refractory prostate cancer cells when combined with TRAIL. Our present data indicate the novel potential of allopurinol as an anticancer agent.

Moreover, as a novel molecular action of allopurinol, we showed that allopurinol up-regulated DR5 and CHOP expression through a transcription. The observation is very surprising because allopurinol functions as a gene expression regulator. Previously, we showed that tunicamycin, an ER stress inducer, up-regulates DR5 and CHOP expression; however, we revealed here that allopurinol regulates the expression in a different manner from ER stress.

Using XO siRNA instead of allopurinol, we showed for the first time that XO knockdown enhances TRAIL-induced apoptosis with increased CHOP and DR5 expression. It has been reported that XO activity is high in brain tumor, lung cancer, colorectal cancer, and damaged tissue but not in normal tissues (28-30); therefore, allopurinol plus TRAIL might specifically induce apoptosis in cancer cells but not in normal cells.

Our study has important implications in cancer treatment. As mentioned above, allopurinol enables to overcome resistance to TRAIL and hormone in prostate cancer. Furthermore, more than a half of all malignant tumors possess an inactivating mutation in the p53 gene and p53 modulates the sensitivity against conventional anticancer agents (31, 32). Because PC-3 and DU145 cells harbored inactivated p53, combined treatment with allopurinol and TRAIL is also useful for p53-deficient tumor cells.

In conclusion, we have shown that allopurinol cooperates with TRAIL and induces apoptosis in prostate cancer cells via DR5 up-regulation caused by the inhibition of XO. These results suggest that the combined treatment of allopurinol with TRAIL may be promising for the treatment of hormone-refractory prostate cancer. At present, effects of anticancer
agent in a single use are limited. Our present data raise a possibility that the combined use of agents that have clinical benefits in other disease expands the ability of each agent and becomes useful for cancer treatment.

Materials and Methods

Reagents

Allopurinol, tunicamycin, and soluble recombinant human TRAIL/Apo2L were purchased from Sigma and PeproTech, respectively. Recombinant human DR5 (TRAIL-R2)/Fc chimera and the caspase inhibitors zVAD-fmk, zDEVD-fmk, zIETD-fmk, zLEHD-fmk, and zAEVD-fmk were purchased from R&D Systems.

Cell Culture

Human prostate cancer cell lines, PC-3 and DU145, were maintained in RPMI 1640 with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2.

Western Blot Analysis

Western blot analysis was done as previously described (33) using rabbit polyclonal anti-DR5 antibody (1:250; Prosci);...
anti-CHOP (1:200) and anti-XO (1:100) antibodies (Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin antibody (1:1,000) were used for detection (Sigma).

**Northern Blot Analysis**

Northern blot analysis was done as previously described using full-length DR5 or CHOP cDNA as a probe (34).

**Plasmid Preparation**

pDR5PF and deletion mutant plasmids containing DR5 promoter were previously described (35). CHOP3K and deletion mutant plasmids containing CHOP promoter were previously described (27). pCHOP/−256, pCHOP/−220, and pCHOP/−150 were generated by self-ligation following Sac I and Sac II digestion and Klenow fragment treatment. pCHOP/mtAP-1 and pCHOP/mtHSF were generated with a site-directed mutagenesis kit (Stratagene).

**Transfection and Luciferase Assay**

A series of DR5 and CHOP reporter plasmids and vacant vector plasmid (1.0 µg) were transfected into PC-3 and DU145 cells (1.5 × 10⁵) using the DEAE-dextran method (CellPhect, GE Healthcare). After 24 h, the cells were treated with or without allopurinol for 24 h and then harvested. Levels of luciferase activity were normalized with protein concentrations. Luciferase assays were carried out in triplicate, and the experiments were repeated several times. Data were analyzed using Student’s t test, and differences between DMSO and allopurinol treatment were considered significant when P < 0.05.

**Determination of Apo2L/TRAIL Receptor Expression**

As previously described (36), cells were harvested by short trypsinization, washed once with ice-cold PBS containing 1% bovine serum albumin, and resuspended in 100 µL PBS with 1% bovine serum albumin. Then, 5 µg of phycoerythrin-labeled

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**FIGURE 7.** Allopurinol enhances CHOP promoter activity via ERSE-independent pathway. A. Structures of CHOP promoter-luciferase reporter plasmids. Circles indicate ERSE. Open circle: wild-type; closed circle: mutant. B and C. Allopurinol enhanced CHOP promoter activity in PC-3 and DU145 cells. Luciferase assay was carried out with PC-3 (B) and DU145 (C) cells treated with 200 µmol/L allopurinol, 1 µg/mL tunicamycin, or DMSO for 24 h after transfection of a luciferase plasmid containing various sizes of CHOP promoters.
anti-Apo2L/TRAIL receptor antibody (DR4, DR5, DcR1, or DcR2; eBioscience) were added. To assess nonspecific staining, phycoerythrin-labeled control IgG isotypes (eBioscience) were applied. After 30-min incubation on ice, cells were washed and 2 × 10⁶ cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson).

Detection of Apoptosis

DNA fragmentation was quantified by the percentage of hypodiploid DNA (sub G₁). PC-3 and DU145 cells were treated with PBS containing 0.1% Triton X-100. Cells were then treated with RNase A (Sigma) and the nuclei were stained with propidium iodide (Sigma). Measurement and analyses were carried out as previously described (37). The DNA content was measured using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson). For all assays, 10,000 cells were counted.

siRNAs

The DR5 and LacZ siRNA sequences were previously described (34, 35). The XO siRNA sequences were as follows

\[
5'-r(GCCCUUUGCUAUGGUGGAA)dTdT \quad \text{and} \quad 5'-r(UUCCACCAUAGCAAAGGCC)-dTdT
\]

(sense and antisense, respectively). 5'-r(GCCCUUUGCUAUG-GUGGAA)dTdT and 5'-r(UUCCACCAUAGCAAAGGCC)-dTdT (synthesized by Sigma). LacZ siRNA was used as a siRNA control. In brief, 1 d before transfection, PC-3 cells were seeded without antibiotics at a density of 30% to 40%. DR5, LacZ, and XO siRNA were transfected into cells using a modified Oligofectamine protocol (Invitrogen), in which the volume of Oligofectamine was reduced to one third of the recommended volume to limit toxic effects. Twenty-four hours after transfection, cells were treated with allopurinol and/or TRAIL for 24 h and then harvested.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
References
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