Lupulone triggers p38 MAPK-controlled activation of p53 and of the TRAIL receptor apoptotic pathway in human colon cancer-derived metastatic cells

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Abstract. We previously reported that the chemopreventive agent lupulone induces apoptosis through activation of the extrinsic pathway via TRAIL DR4/DR5 death receptors overcoming SW620 cell resistance to TRAIL. However, the underlying molecular mechanisms remain unknown. Since the mitogen-activated protein kinases (MAPks), Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 control fundamental cellular processes such as apoptosis, we determined the role of these MAPks in lupulone-triggered apoptosis. We analyzed the effects of JNK, ERK and p38 MAPK inhibitors on lupulone-induced apoptosis by flow cytometry using specific antibodies and real-time RT-PCR. Our data showed that among the MAPks, only p38 played a major role in lupulone-triggered apoptosis. In contrast to JNK and ERK inhibition, the specific inactivation of p38 inhibited the lupulone-triggered up-regulation of p53 and TRAIL-death receptor DR4/DR5 expression, and prevented DNA fragmentation. Lupulone treatment enhanced the expression of the anti-apoptotic Mcl-1 protein by 60% favoring the preservation of mitochondrial integrity. The inactivation of p38 initiated a 50% reduction in Mcl-1, Bcl-2 and Bax expression without changing the Mcl-1/Bax ratio suggesting that p38 was not involved in the protective effect of lupulone on mitochondria. Our data support the view that the lupulone-triggered enhanced expression of p38 plays a major role in the activation of p53 and of the TRAIL-death receptor apoptotic pathway in SW620 human colon cancer-derived metastatic cells.

Introduction

Family members of the mitogen-activated protein kinases (MAPK) regulate and control fundamental cellular processes such as growth, proliferation, differentiation, migration or/and apoptosis. Distinct groups of MAPks have been described in mammals: extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 isoforms (1). The ERK signaling pathways are activated in response to growth factors or mitogenic signals and play a key role in cell proliferation, survival and differentiation.

Activation of ERK induces the amplification and deregulation of its nuclear transcription targets, c-myc and AP-1 (2,3). It is clear that the deregulation of these pathways can lead to many other perturbations at the level of cell signaling depending on the cell type and stress signal (4,5). The JNK and p38 MAPK pathways are described as stress-activated kinases, inducing inflammation and in certain cases programmed cell death. The JNK pathway is activated by cytokines, UV radiation, growth factor deprivation or DNA-damaging agents while p38 isoforms are activated by environmental stress and inflammatory cytokines (1). In response to stress stimuli, JNK as well as p38 MAPK may enhance p53 expression, p53 stabilization and consequently p53-induced apoptosis. Furthermore, it was also reported that p38 activation induces the translocation of the pro-apoptotic Bax protein from the cytoplasm to mitochondria while JNK may phosphorylate the anti-apoptotic Bcl-2 protein leading to its inactivation (5,6). However, the precise regulatory mechanisms involved in the apoptotic effects triggered by these kinases still remain unclear.

It was shown that MAPK signaling pathways are disregulated in cancer cells altering their apoptotic response to antitumor agents. The roles played by MAPK are strongly context-dependent, influenced by cell type, nature and duration of stimuli in order to monitor apoptosis or cell survival (7). Thus, the participation of ERK, JNK or p38 in the response of tumor cells to antitumor agents is very complex, and both destructive and protective roles of each MAPK have been proposed in different systems (5,8,9). It was also reported that activation of JNK or p38 often leads to the suppression of ERK activity modulating the apoptotic responses in different cancer cell lines (10,11).

We reported previously that lupulone, a β-acid largely present in hops (Humulus lupulus L), is able to activate the
extrinsic apoptotic signaling pathway through the up-regulation of p53 and of TRAIL death-receptor apoptotic pathways in TRAIL-resistant metastatic SW620 cells (12,13). Here we aimed to investigate the role of ERK, JNK and p38 kinases in these events.

Materials and methods

Lupulone. Lupulone was obtained from an industrial by-product that contains high amounts of β-acids (Carlsberg Breweries A/S, Strasbourg, France) and was isolated following the procedure described previously (14).

Cell culture. SW620 cells were obtained from the European Collection of Animal Cell Culture (Salisbury, UK). They were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and supplemented with 10% heat-inactivated (56°C) horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids (Invitrogen Corp., Cergy Pontoise, France) and maintained at 37°C in a humidified atmosphere with 5% CO2. For experiments, after trypsinization (0.5% trypsin/2.6 mM ethylenediamine tetra-acetic acid), cells were seeded at 1x10⁶ cells in culture dishes (100 mm internal diameter). The culture medium was DMEM supplemented with 3% heat-inactivated horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml transferrin, 5 ng/ml selenium, 10 µg/ml insulin and 1% non-essential amino acids (Invitrogen Corp.).

Cell death analysis. SW620 cells (1x10⁶) were seeded in culture dishes and pre-treated with JNK-specific inhibitor SP600125 (10 µM) or ERK-specific inhibitor, SL327 or p38-specific inhibitor, SB203580 (10 µM) (Sigma-Aldrich, Munich, Germany) 30 min before lupulone treatment (40 µg/ml). Cells were harvested by trypsinization at 24 and 48 h, washed with PBS, centrifuged and fixed with 1 ml methanol:PBS (9:1 v/v) for 1-3 h at -20°C. Cells were washed twice in PBS and fixed with 1 ml PBS and incubated with FITC-conjugated mouse anti-human p53 antibody (Calbiochem; Merck Chemicals, France) or FITC-conjugated mouse IgG1 monoclonal isotype control antibody (BD Biosciences) for 1 h at 4°C in the dark. After washing with a solution of PBS/BSA 0.2%/Tween 0.5%, cells were re-suspended in PBS (37°C), and the fluorescence (515 nm) of 10,000 events per sample was analyzed by FACScan and CellQuest software.

Expression of TRAIL receptors DR4 and DR5 after p38 inhibition. Cells were pre-treated for 30 min with the p38 inhibitor SB203580 at 10 µM and treated with lupulone (40 µg/ml) at 24 and 48 h. After trypsinization, cell pellets were washed with PBS and incubated with monoclonal mouse anti-human antibodies TRAIL-R1 (DR4) (1:100) or TRAIL-R2 (DR5) (1:100) (Alexis Biochemicals, Lausen, Switzerland) for 30 min at 4°C. Cells were washed twice with PBS and incubated with FITC-conjugated goat anti-mouse IgG1 antibody (1:50) (AbD Serotec, Düsseldorf, Germany) or with FITC-conjugated mouse IgG1 monoclonal isotype control antibody (1:50) for 30 min at 4°C in the dark. After washing with PBS, cells were re-suspended in PBS, and the fluorescence of 10,000 events per sample (515 nm) was analyzed by FACScan and CellQuest software.

Expression of Bcl-2, Mcl-1 and Bax after p38 inhibition. SW620 cells were pre-treated for 30 min with the p38 inhibitor SB203580 at 10 µM and treated with lupulone (40 µg/ml) for 24 and 48 h. Cells were harvested by trypsinization and proceeded to the fixation (PBS/PFA 4%) and permeabilization (PBS/BSA 0.2%/tween 0.5%) step. For Bcl-2 protein detection, cells were labelled directly with 20 µl FITC-conjugated mouse anti-human Bcl-2 monoclonal antibody or FITC-conjugated mouse IgG1 monoclonal isotype control antibody for 30 min at 4°C in the dark. For Mcl-1 and Bax detection, cells were incubated with rabbit anti-human Bax polyclonal antibody or rabbit anti-human Mcl-1 polyclonal antibody (1:100) (BD Biosciences) for 30 min at 4°C. After washing twice, FITC-conjugated swine anti-rabbit F(ab″)2/FITC antibody was added (1:10) (Abcam, Paris, France) for 30 min at 4°C in the dark. After washing twice in permeabilization buffer, the fluorescence of 10,000 cells was analyzed using FACScan flow cytometer and CellQuest software.

Analysis of mRNA levels by RT-PCR. To determine expression of DR4, DR5 and p53 mRNA, extraction of total RNA and analysis by RT-PCR were performed after treatment with the p38 inhibitor SB203580 and lupulone. Total RNA was extracted using the RNeasy Mini kit (Qiagen, VWR, Copenhagen, Denmark) following the manufacturer's instructions. The High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, USA) was used to reverse transcribe RNA (1 µg) in 20 µl of reaction mix, and measurements of the transcription levels of the selected genes were performed with TaqMan Gene Expression assays (protein p53, Hs00153349; DR4, Hs00269492; DR5, Hs00366272; Applied Biosystems). β-actin was assigned as an endogenous control (cat. no. Hs99999903; Applied Biosystems). Real-time RT-PCR was performed with TapMan Universal PCR master mix and ABI Prism 7500 Sequence Detection System (Applied Biosystems Sequence detector) in triplicate wells. The data were analyzed with a comparative threshold cycle (Ct) method. This method determines the values of Δ cycle threshold (ΔCt) by normalizing the average Ct value of each treatment with the value of each opposite endogenous control (β-actin). Then, calculation of 2^-ΔΔCt of each treatment and statistical analysis were performed as described by Livak and Schmittgen (15).

Detection of p53 expression by cell flow cytometry. Cells were treated with p38 inhibitor, SB203580 (10 µM) 30 min before the treatment with lupulone (40 µg/ml). Cells were harvested by trypsinization at 24 and 48 h, cell pellets were washed with phosphate-buffered saline (PBS) and fixed with a solution of PBS containing 4% paraformaldehyde (PFA) for 1 h at 4°C in the dark. Cell pellets were washed with a solution of PBS/BSA 0.2%/Tween 0.5%, cells were re-suspended in PBS (37°C), and the fluorescence (515 nm) of 10,000 events per sample was analyzed by FACScan and CellQuest Software.
pellets were washed with a solution of PBS containing 0.2% BSA and were fixed in a solution of PBS/4% PFA for 1 h at 4°C in the dark. Cells were washed twice in PBS/0.2% BSA, and pellets were permeabilized with a solution of PBS/0.5% Tween-20/0.2% BSA for 30 min. After washing, cell pellets were incubated with the Mebstain Apoptosis direct kit solution (MBL, Japan) for 90 min at 37°C, in the dark. This solution contained TdT buffer II, FITC-dUTP and TdT (transferase) (18:1:1). Negative control cells were incubated with a solution of TdT buffer and FITC-dUTP (19:1), and H2O2 was used as a positive control. Cells were washed twice, were re-suspended in PBS/0.2% BSA and the fluorescence of the cells (10,000 events) was analyzed by flow cytometry and CellQuest software.

Statistical analysis. All data were presented as the mean ± standard error (SE) from three independent experiments. Significant differences between the control and treated groups were evaluated by one-way ANOVA analysis, Student's t-test, or the Student-Newman-Keuls multiple comparison test was used to determine the significance of statistical differences between data.

Results

Inhibition of MAPKs and SW620 cell survival. In order to determine the role of JNK, ERK and p38 MAPKs in lupulone-triggered SW620 cell death, the cells were treated with specific inhibitors of JNK (SP600125), ERK (SL327) and p38 (SB203580) (16-18), at a final concentration of 10 µM for 30 min before lupulone treatment (+lup) (40 µg/ml) for 24 and 48 h. At each time point, cells were harvested, stained with propidium iodide and analyzed by flow cytometry. The percentage of hypodiploid cells present in the sub-G0/G1 region is represented as a histogram and corresponds to the mean value ± SE of at least three separate experiments. For each time period, 24 or 48 h, columns not sharing the same superscript letter differ significantly; a≠b≠c≠d, P<0.05.

Up-regulation of p53 expression by p38 after lupulone treatment. In response to stress, p38 may favor apoptosis by regulating p53 protein expression (1-20). In SW620 cells, p53 protein carries two mutations which appear not to affect its DNA binding ability (20). In order to determine a possible cross-talk between p38 and p53 regulation, levels of p53 transcript and protein expression were assessed using real-time PCR and flow cytometry. As shown in Fig. 2A, the amount of p53 transcripts was up-regulated after lupulone treatment in a time-dependent manner reaching a 7-fold increase over untreated cells (ctrl). Data were calculated with the comparative cycle threshold method (15) and correspond to the mean value ± SE of at least three separate experiments. For 24 or 48 h, "P<0.001. (B) Determination of p53 protein expression by flow cytometry. At each time point, cells were harvested and stained with FITC-conjugated anti-human p53 antibody. Increased green fluorescence was measured by flow cytometry, and data are represented by a histogram as the percentage of cells expressing p53 protein. Data correspond to the mean value ± SE of at least three separate experiments. For 24 or 48 h, columns not sharing the same superscript letter differ significantly; a≠b≠c and a'≠b'≠c', P<0.05.

(Fig. 1). These data suggest a specific role of p38 signaling in lupulone-triggered cell death.
Effect of p38 inhibition on TRAIL death receptors DR4/DR5.
To determine the potential role of p38 MAPK on the activation of TRAIL death receptor expression at the cell surface observed after lupulone treatment in SW620 cells, the levels of TRAIL DR4 and DR5 mRNA and protein expression were assessed using real-time PCR and flow cytometry.

As illustrated in Fig. 3A and B, specific inhibition of p38 prevented the lupulone-triggered up-regulation of both TRAIL DR4/DR5 mRNA and protein expression. These data indicate that p38 MAPK was directly involved in the regulation of the extrinsic apoptotic TRAIL signaling pathway after lupulone treatment.

Effect of p38 inhibition on Bcl-2, Mcl-1 and Bax expression.
We previously showed that in response to lupulone, SW620 cells were able to activate mechanisms favoring the protection of mitochondrial function (12,13). Since it was reported that anti-apoptotic proteins of the Bcl-2 family such as Bcl-2 or Mcl-1 are able to stabilize the mitochondrial membrane (22), we investigated whether p38 activation by lupulone was able to modulate the expression of these anti-apoptotic proteins. Using flow cytometry, we measured the percentage of cells expressing anti-apoptotic Bcl-2, Mcl-1 or pro-apoptotic Bax proteins after inhibition of p38 expression in lupulone-treated cells.

After lupulone treatment for 48 h, both Bcl-2 and Bax expression was increased in the same proportions (Fig. 4). The number of cells expressing the anti-apoptotic protein Mcl-1 was enhanced by 60%, and the Mcl-1/Bax (anti-apoptotic/pro-apoptotic protein) ratio was equal to 2. The inhibition of p38 function caused a 50% reduction in lupulone-triggered activation of Mcl-1, Bcl-2 and Bax. However, the Mcl-1/Bax ratio remained unchanged (equal to 2) in the presence and in the absence of the p38 inhibitor.

Involvement of p38 MAPK in lupulone-triggered DNA fragmentation.
We previously reported that lupulone induced the extrinsic apoptotic pathway involving the activation of the caspase-8 and caspase-3 cascade (12). Apoptosis is accompanied by nuclear condensation and fragmentation with extensive degradation of chromosomal DNA, these events being a biochemical hallmark of apoptosis (23). The TdT-mediated dUTP-biotin nick-end labeling (TUNEL) method which was developed by Gavrieli et al (24) is a sensitive method to visualize in situ the DNA fragmentation at the single cell level. In lupulone-treated SW620 cells for 48 h, the inhibition of p38 function caused a 65% reduction in the number of cells with fragmented DNA (Fig. 5). These data strongly suggest that p38 may represent a major signaling pathway in the induction of apoptosis after lupulone treatment.

Discussion
The role of the MAPK signaling pathway has been reported to be important in regulating apoptosis during conditions of
We previously reported that lupulone induced apoptosis by an activation of the extrinsic pathway via TRAIL-DR4/DR5 death receptors overcoming SW620 cell resistance to TRAIL (12). Furthermore, we showed that the lupulone-triggered activation of p53 in SW620 cells plays a pro-apoptotic role through the up-regulation of TRAIL-death receptor transcripts and protein (13). However, the molecular mechanisms by which lupulone activated p53 expression and TRAIL DR4/DR5 receptors remain unknown.

Our present data showed that when SW620 cells were exposed to lupulone in the presence of specific inhibitors of JNK, ERK or p38 MAPK, only the specific inhibition of p38 was able to prevent the apoptotic effects of lupulone in these cells. Most studies have reported that p38 MAPK pathways are involved in a variety of complex cellular responses (26). Similar to JNK, p38 may regulate apoptosis, cell cycle progression, growth and differentiation (1,27). Two main MAPKK can be activated by p38: MKK3 and MKK6. Activation of MKK6 can induce p38 expression leading to promotion of caspase activity and cell death (28). Other studies have demonstrated the prominent role of p38 in the induction of Bax translocation to mitochondria leading to mitochondrial disruption and cell death (6,29,30).

The transcription factor p38 is often involved in p53-regulated apoptosis and acts as a negative regulator of cell cycle progression (31,32). In SW620 cells, p53 protein carries two mutations, which do not affect its ability to bind to DNA (21). In the present study we show that, in spite of these mutations, transcription and protein expression of mutated-p53 were up-regulated by p38 in lupulone-treated SW620 cells. We also showed that the activation of TRAIL-DR4/DR5 death receptors by lupulone was p38-dependent. Indeed, the specific inhibition of p38 prevented DR4 and DR5 gene transcription and related protein synthesis. Our data suggest that lupulone may overcome TRAIL resistance of SW620 cells by activating p38, which in turn up-regulates the transcription and protein expression of p53 and TRAIL-DR4/DR5 death receptors.

We previously demonstrated that lupulone activated the TRAIL-receptor apoptotic pathway (extrinsic pathway) leading to an activation of the caspase-8 caspase-3 cascade (12). We also reported a cross-talk between the extrinsic and mitochondrial (intrinsic) apoptotic pathways through a caspase-8 dependent cleavage of Bid protein causing mitochondrial perturbations. However, mitochondrial function was not compromised since no release of cytochrome c was observed (12,14). Thus mitochondrial functions remained preserved in lupulone-treated SW620 cells. In the present study we showed that lupulone induced the activation of the survival factor Mcl-1, which is a key factor in mitochondrial protection (22). The specific inhibition of p38 caused a 50% decrease in Mcl-1, Bcl-2 and Bax expression. The Mcl-1/Bax (survival/pro-apoptotic factors) ratio remained unchanged after p38 inactivation suggesting that p38 may not play a major role in the protection of mitochondrial integrity. Our data highlight the specificity of p38 in activating the extrinsic apoptotic pathway (death-receptor pathway) in metastatic SW620 cells in response to lupulone. The key role of p38 in apoptotic processes triggered by lupulone was confirmed by the observation that DNA fragmentation was prevented in these cells by the p38 inhibitor.

Figure 5. Effect of p38 inhibition on cellular DNA fragmentation induced by lupulone. Cells were pre-treated with p38 inhibitor, SB203580 (10 μM), 30 min before lupulone treatment (40 μg/ml) for 24 and 48 h. At each time point, cells were harvested, fixed, permeabilized and stained with the Mebstain Apoptosis direct kit solution (MB l, Japan), as described in Materials and methods, and analyzed by flow cytometry. H2O2 was used as a positive control. Cells with increased green fluorescence produced DNA fragmentation. (A) Data are presented as representative histogram plots and the shift on the right corresponds to cells with fragmented DNA. Negative control and control cells presented the same profile (data not shown). (B) Histogram depicts the percentage of cells with fragmented DNA. Data are the mean value ± SE of at least three separate experiments. At each time period (24 or 48 h) columns not sharing the same superscript letter differ significantly; a≠b≠c and a’≠b’≠c’, P<0.05.
In conclusion, this study provides new insights into the complex mechanisms involved in lupulone-triggered apoptosis in TRAIL-resistant SW620 cells. We showed here that the chemopreventive agent lupulone activated the p38 signaling pathway which played a major role in the up-regulation of TRAIL death receptors and of p55 expression at the genetic and protein levels. As shown previously (12), TRAIL DR4 and DR5 receptors activated caspase-8, and consequently caspase-3 leading to the observed DNA fragmentation. It has been reported that other chemopreventive compounds such as curcumin, resveratrol or quercetin induced apoptosis through an activation of JNK and p38 pathways and notably in SW620 cells after berberine treatment (33,34). Our data support the view that the lupulone-triggered enhanced expression of p38 plays a major role by controlling p53 and the TRAIL-receptor apoptotic pathway in metastatic SW620 cells.

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References