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The role of p38 MAP kinase in the synergistic cytotoxic action of calcitriol and TNF-α in human breast cancer cells☆

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Abstract

Calcitriol, the hormonal form of Vitamin D, potentiates the activity of some agents of the anti-cancer immune system including tumor necrosis factor-α (TNF-α). Different signaling pathways activated by TNF-α may be targets for calcitriol action. Activation of p38 MAP kinase was shown to have both pro- and anti-apoptotic actions in TNF-α-induced programmed cell death depending on cell context. Treatment of MCF-7 breast cancer cells with TNF-α resulted in activation of p38 MAP kinase that persisted for at least 24 h. Whereas calcitriol had no effect on the earlier phase of p38 MAP kinase activation (up to 1 h), it inhibited the activation of this pathway between one and 24 h after exposure to TNF-α. Both calcitriol and the p38 MAP kinase inhibitor SB203580 enhanced TNF-α-induced cytotoxicity and drop in mitochondrial membrane potential, but their combined effect was sub-additive. Taken together, these findings suggest that p38 MAP kinase plays an anti-apoptotic role in TNF-α-induced cytotoxicity in MCF-7 cells and that the synergistic interaction between TNF-α and calcitriol, leading to mitochondrial damage and subsequent cell death, is partially due to modulation of this signaling pathway.

Keywords: Vitamin D; TNF-α; p38 MAP kinase; Mitochondria

1. Introduction

Activation of p38 MAP kinase may play opposite roles in tumor necrosis factor-α (TNF-α)-induced apoptosis depending on the cell type. On the one hand, inhibition of p38 MAP kinase reduced TNF-α-induced apoptosis of primary brown fetal rat adipocytes [1] and endothelial cells from murine liver sinusoids [2], and on the other hand, activation of p38 MAP kinase protected various cell types such as PC12 [3], U937 [4], NIH-3T3 [5] and bovine endothelial [6] cells challenged by the cytokine.

The ability of calcitriol, the hormonal metabolite of Vitamin D_3, to enhance the cytotoxic activity of several anti-cancer agents has been demonstrated in different experimental models [7]. We have previously reported that calcitriol enhanced TNF-α-induced programmed cell death and that impairment of mitochondrial metabolism, as manifested by drop of mitochondrial membrane potential and cytochrome c release, is a central event in this process [8,9]. In this study, we examined the notion that activation of p38 MAP kinase partakes in the interaction between calcitriol and TNF-α. We found that in breast cancer cells p38 MAP kinase has an anti-apoptotic role during TNF-α-induced programmed cell death and that the pro-apoptotic action of calcitriol in this system may be partially due to inhibition of the p38 MAP kinase pathway.

2. Material and methods

2.1. Materials

Tissue culture media were purchased from Biological Industries (Beit Haemek, Israel). Tissue culture plasticware were from Corning Glass Work (Corning, NY), except for clear-bottom black 96-well microtiter plates obtained from Greiner Labortechnik (Kremsmuenster, Germany). Calcitriol was obtained from Hoffman-LaRoche (Nutley, NJ, the generous gift of Dr. M. Uskokovic). Human recombinant TNF-α was obtained from PeproTech (Rocky Hill, NJ). Crystal violet (CV) was from Edward Gurr (London, UK). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), was from ALEXIS Biochemicals (Lausen, Switzerland). Anti p–p38 monoclonal antibody was from Cell Signaling Technology Inc. (Beverly,

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CA). Peroxidase-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). All other reagents were of analytical grade.

2.2. Cell culture

MCF-7 breast cancer cells were maintained in Dulbecco's modified Eagle medium containing 4.5 g/l glucose supplemented with 10% fetal calf serum and antibiotics. For all experiments, cultures were treated with calcitriol and/or TNF-α 24 h after seeding. The vehicle of calcitriol, ethanol, was added to control cultures, and its concentration was kept at or below 0.06%.

2.3. Cell viability measurement

Cells were quantified by CV staining as previously described [10]. The cytotoxic effect of TNF-α was calculated as follows: cytotoxicity (%) = [1 − (CV staining in 'treated wells'/CV staining in 'control wells')] × 100 'control wells' were treated identically to wells containing TNF-α ('treated wells') except for the addition of the cytokine.

2.4. Mitochondrial membrane potential assay

Mitochondrial membrane potential was monitored as previously described [9]. MCF-7 cells were seeded in black, clear-bottom 96-well microtiter plate (10^4 cells per well) and treated with calcitriol (100 nM) for 24 h. TNF-α (10 ng/ml) and SB203580 (SB, 1 μM) were added 12 h before the assay of mitochondrial membrane potential. Mitochondrial membrane potential was assayed by using the cationic dye JC-1 that undergoes potential-dependent accumulation in the mitochondria. It emits green (ex. 490 nm, em. 540 nm) fluorescence from mitochondria with low (below 140 mV, [11]) membrane potential and red fluorescence (ex. 540 nm, em. 590 nm) from mitochondria with high membrane potential. Fluorescence was quantified by a fluorescence plate reader (FLUOstar, BMG LabTechnologies, Offenburg, Germany). Changes in the ratio between the measured red and green fluorescence intensities are indicative of changes in the mitochondrial membrane potential. This ratio was calculated for each culture after subtraction of fluorescence intensity obtained in wells containing medium and serum without cells.

2.5. Western blot analysis

Cells were washed with ice-cold PBS, lysed with SDS-sample buffer and boiled for 15 min. Samples were centrifuged before electrophoresis and subjected to SDS-PAGE under reducing conditions using 10% polyacrylamide gels (20 μg protein per lane). Proteins were transferred to nitrocellulose membranes and probed with anti dually phosphorylated p38 antibody. Detection was carried out by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence [12]. The content of protein in cell extracts was measured by the BCA Protein Assay Kit (Pierce Biotechnology Inc., IL).

3. Results

First we examined the time course and extent of p38 MAP kinase activation following exposure of MCF-7 cells to TNF-α. Fig. 1A shows that TNF-α triggered the activation of p38 MAP kinase that was apparent already after 15 min of treatment. Thereafter, the extent of activation declined, but did not reach control levels even 4 h after the addition of the cytokine. Pretreatment with calcitriol for 24 h prior to TNF-α reduced the extent of p38 MAP kinase activation 1, 2 and 4 h following exposure to TNF-α (Fig. 1A) Similar results were obtained in cultures treated for 24 h with TNF-α (Fig. 1B). It is noteworthy that calcitriol did neither affect p38 MAP kinase basal activity, nor the early phase of p38 MAP kinase activation, 15 minutes after addition of TNF-α (Fig. 1A).

In order to assess the role of p38 MAP kinase activation in TNF-α-induced apoptosis, we employed the specific p38 MAP kinase inhibitor SB203580. As previously reported [13,14], we also found this inhibitor to decrease the extent of dual phosphorylation of p38 MAP kinase (Fig. 2A). Addition of the inhibitor sensitized MCF-7 cells to TNF-α action (Fig. 2B). It may thus be inferred that p38 MAP kinase protects MCF-7 cells from TNF-α-induced cell death. As seen in Fig. 2B, the ability of calcitriol to enhance TNF-α-induced cell death is reduced in the presence of SB203580, in accordance with a shared mechanism of action. Since calcitriol did not inhibit the early phase of p38 MAP kinase activation, but affected the activation at later times (Fig. 1A), we assessed the importance of this late action by adding SB203580 as late as 4 h after the beginning of TNF-α treatment. We found that such treatment was as effective in

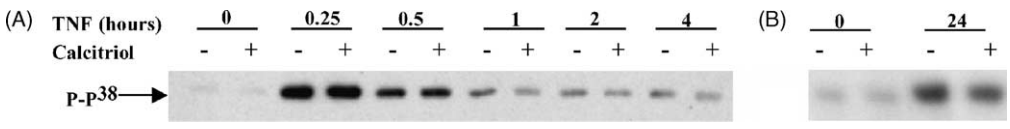

Fig. 1. The effect of TNF-α and calcitriol on p38 MAP kinase activation. MCF-7 cells were seeded in 3.5 cm petri dishes (2×10^5 cells per dish) and harvested 48 h later. Cells were treated with calcitriol (100 nM) 24 h before harvesting. Activation of p38 MAP kinase was detected at earlier (A) and later (B) times following exposure to TNF-α by immunoblotting with anti dually phosphorylated p38 antibody as described in Section 2.

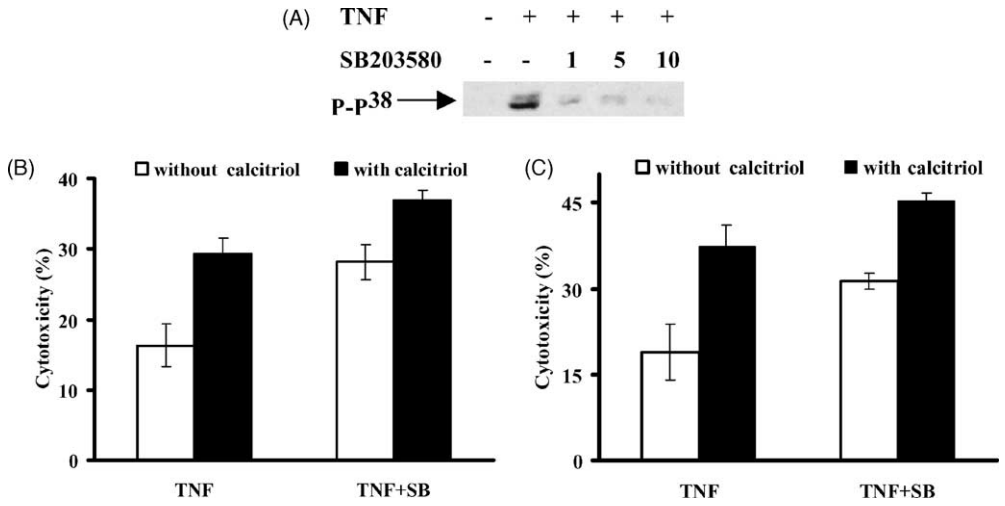

Fig. 2. The effect of calcitriol on TNF-α-induced cytotoxicity in the presence and absence of p38 MAP kinase inhibitor. MCF-7 cells were seeded in 3.5 cm Petri dishes (2×10^5 cells per dish, A) and 96-well microtiter plates (7000 cells per well, B and C). For the assessment of its enzyme inhibitory activity, the p38 inhibitor (SB) was added to cells 30 min before the addition of TNF-α (10 ng/ml) for additional 15 min (A). For cytotoxicity assay, 24 h after seeding cells were treated with TNF-α (1 ng/ml) for 48 h in the presence or absence of calcitriol (100 nM). SB203580 (1 μM) was added 30 min before (B) or 4 h after (C) TNF-α. Cytotoxicity was assessed by CV staining as described in Section 2. Data are expressed as the mean ± S.E.M. of four independent experiments (B), each with five parallel cultures or as the mean ± S.D. of five parallel cultures (C). The significance of the difference between groups was assessed by paired (B) and unpaired (C) Student's *t*-test. $P < 0.05$ (TNF-α vs. TNF-α with calcitriol); $P < 0.05$ (groups treated with SB vs. without SB).

potentiating TNF-α-induced cytotoxicity as simultaneous addition of the inhibitor and TNF-α. Taken together, our findings suggest that the enhancing effect of calcitriol may be at least partially explained by the moderate inhibition of p38 MAP kinase activated by TNF-α.

Table 1
The effect of calcitriol on TNF-α-induced drop in mitochondrial membrane potential in the presence and absence of p38 MAP kinase inhibitor

	Red/green fluorescence	
	Without calcitriol	With calcitriol
A		
Control	3.15 ± 0.27	2.99 ± 0.18
TNF-α	2.57 ± 0.18[a]	1.68 ± 0.11[b]
TNF-α + SB	2.23 ± 0.22[c]	1.57 ± 0.14[b]
Number of experiment	$R_{-\text{calcitriol}} - R_{+\text{calcitriol}}$	
	Without SB	With SB
B		
1	0.89	0.66
2	0.91	0.54
3	0.88	0.74
Average	0.89 ± 0.01	0.65 ± 0.1[d]

The detection of mitochondrial membrane potential changes was performed as described in Section 2. (A) Data are expressed as mean ± S.D. of five parallel cultures. The significance of the difference between groups was assessed by unpaired Student's *t* test. The letters (a) $P < 0.01$, TNF-α vs. control; (b) $P < 0.001$, with calcitriol vs. without calcitriol; (c) $P < 0.05$, TNF-α vs. TNF-α with SB. (B) Data expressed as the delta between the ratios of red/green fluorescence (*R*) in control and calcitriol-treated cultures. Statistical significance was judged by paired Student's *t*-test ((d) $P < 0.05$).

Next, the combined action of calcitriol and the SB203580 on TNF-α-induced drop in mitochondrial membrane potential was examined. Treatment of MCF-7 cells with TNF-α for 12 h induced a marked decrease in mitochondrial membrane potential ($\Delta\Psi$) (Table 1). Neither calcitriol nor SB203580 on their own affected $\Delta\Psi$ but both of them considerably potentiated the drop of membrane potential induced by TNF-α. However, the net effect of calcitriol was attenuated in the presence of SB203580 (Table 1). The inhibition of p38 MAP kinase by calcitriol may thus partially account for its potentiating effect on the cytokine-mediated impairment of mitochondrial function.

4. Discussion

The interaction of TNF-α with its receptor initiates both pro- and anti-apoptotic signaling pathways. The balance between them determines the final outcome: survival or death. Activation of p38 MAP kinase occurs in the earlier stages of TNF-α-induced apoptosis. Depending on the cellular context, p38 MAP kinase may enhance or inhibit cell death. In our experimental system, inhibition of p38 MAP kinase markedly potentiated both TNF-α-induced cytotoxicity and drop in $\Delta\Psi$ (Fig. 2, Table 1). These findings imply that in MCF-7 cells treated with TNF-α p38 MAP kinase plays an anti-apoptotic role. The protective effect of p38 MAP kinase has been attributed to two mechanisms. (A) Activation of MAPK-activated protein kinase-2 that phosphorylates heat shock protein 27 (HSP27) [15–17] that in turn inhibits procaspase-3 activation by sequestering both cytochrome c

and procaspase-3 [18,19]. (B) Activation of Akt kinase [20] leading to degradation of I-κB, the inhibitor of NF-κB [21] that is known to inhibit TNF-α-induced apoptosis.

We and others have previously shown that calcitriol enhances TNF-α-induced programmed cell death. The aim of this research was to assess the role of p38 MAP kinase in the cross-talk between the cytokine and the hormone. Its role was substantiated by three findings: (A) TNF-α triggered sustained p38 MAP kinase activation, the later phase of which was partially inhibited by pretreatment with calcitriol; (B) addition of the specific p38 inhibitor, SB203580, as late as 4 h after the exposure to TNF-α was sufficient to sensitize the cells to TNF-α-induced cytotoxicity; (C) the net effect of calcitriol on TNF-α-induced cell death and drop in mitochondrial membrane potential decreased in the presence of SB203580. Taken together, these findings indicate that the synergistic interaction of calcitriol with TNF-α at the level of mitochondrial damage and cell death is partially due to inhibition of the p38 MAP kinase signaling pathway.

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