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Mitogen-Activated Protein Kinase Pathway Mediates DBP–maf-Induced Apoptosis in RAW 264.7 Macrophages

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Abstract Vitamin D-binding protein–macrophage-activating factor (DBP–maf) is derived from serum vitamin D binding protein (DBP) by selective deglycosylation during inflammation. In the present study, we investigated the effect of DBP–maf on RAW 264.7 macrophages and the underlying intracellular signal transduction pathways. DBP–maf increased proapoptotic caspase-3, -8, and -9 activities and induced apoptosis in RAW 264.7 cells. However, DBP, the precursor to DBP–maf did not induce apoptosis in these cells. Cell cycle analysis of DBP–maf-treated RAW 264.7 cells revealed growth arrest with accumulation of cells in sub-G_0/G_1 phase. We also investigated the role of mitogen-activated protein kinase (MAPK) pathways in the DBP–maf-induced apoptosis of RAW264.7 cells. DBP–maf increased the phosphorylation of p38 and JNK1/2, while it decreased the ERK1/2 phosphorylation. Treatment with the p38 MAPK inhibitor, SB202190, attenuated DBP–maf-induced apoptosis. PD98059, a MEK specific inhibitor, did not show a significant inhibition of apoptosis induced by DBP–maf. Taken together, these results suggest that the p38 MAPK pathway plays a crucial role in DBP–maf-mediated apoptosis of macrophages. Our studies indicate that, during inflammation DBP–maf may function positively by causing death of the macrophages when activated macrophages are no longer needed at the site of inflammation. In summary, we report for the first time that DBP–maf induces apoptosis in macrophages via p38 and JNK1/2 pathway. J. Cell. Biochem. 90: 87–96, 2003.

Key words: macrophage activation; vitamin D-binding protein (DBP); vitamin D-binding protein–macrophage-activating factor (DBP–maf); RAW 264.7 macrophages; apoptosis; mitogen-activated protein kinase; p38; ERK1/2; JNK1/2; inflammation

Vitamin D-binding protein (DBP) also known as Gc-globulin, is a multi-functional protein present in blood and other body fluids. DBP binds vitamin D metabolites and aids in their transportation. In addition, DBP binds fatty acids G-actin and C5desArg, a chemotactic agent [reviewed in Cooke and Haddad, 1989; Haddad, 1995; Ray, 1996]. DBP has been shown to bind to megalin and cubulin, which assist in renal reuptake of DBP-bound vitamin D metabolites [Nykjaer et al., 1999, 2001]. Furthermore, selective deglycosylation of DBP by B- and T-lymphocyte membrane bound glycosidases results in DBP–macrophage-activating factor (DBP–maf), which serves as a cytokine in activating macrophages and osteoclasts [Yamamoto and Homma, 1991; Yamamoto et al., 1991; Schneider et al., 1995].

DBP has been shown to play a role in inflammation by activating macrophages [Yamamoto and Homma, 1991; Yamamoto et al., 1991]. Activation by a number of macrophage-activating factors facilitates the action of macrophages [Fedorko, 1999]. Mitogen-activated protein kinases (MAPKs) have been shown to play an integral role in macrophages activation [Franklin and McCubrey, 2000]. MAPKs are a group of serine/threonine-specific protein kinases that

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are activated by a wide spectrum of extracellular stimuli. They are important mediators involved in the intracellular network of interacting proteins that transduce extracellular signals to intracellular responses [Su and Karin, 1996]. To date, three distinct classes of MAPKs: the extracellular signal-regulated kinases (ERK1/2), stress-activated protein kinases/c-Jun NH2-terminal kinase (JNK1/2), and p38 MAPK have been identified [Su and Karin, 1996]. Activation of ERKs by a variety of growth factors and hormones is associated with cell proliferation [Robinson and Cobb, 1997]. In contrast, the JNKs and p38 MAPKs are activated by environmental stresses, such as UV radiation, heat and osmotic shock, protein synthesis inhibitors, and lipopolysaccharide, and proinflammatory cytokines [Su and Karin, 1996; Whitmarsh and Davis, 1996; Ip and Davis, 1998]. Several studies associate JNK or p38 activation with apoptosis [Verheij et al., 1996; Kawakami et al., 1997; Kummer et al., 1997], whereas activation of ERK seems to be associated with cell proliferation [Carter et al., 1998; Wang et al., 1998]. A balance between ERK activation relative to active JNK and/or p38 determines the fate of a cell [Xia et al., 1995].

DBP is converted to a potent macrophage-activating factor, DBP–maf by membrane bound inducible isoforms of α-mannosidase, β-galactosidase, and sialidase of B- and T-lymphocytes. The events responsible for the conversion of DBP to DBP–maf were demonstrated in an in vitro model using peritoneal cells containing adherent macrophages and nonadherent B- and T-cells [Ngwenya and Yamamoto, 1990]. DBP can also be converted to DBP–maf by the treatment of DBP with purified β-galactosidase, α-mannosidase and sialidase [Yamamoto and Kumashiro, 1993]. Treatment of peritoneal macrophages with DBP–maf resulted in an increase in phagocytosis and superoxide production by macrophages. Similarly, treatment of rats with DBP–maf also increased the superoxide production by peritoneal macrophages [Yamamoto and Homma, 1991; Yamamoto et al., 1991]. In recent reports, DBP–maf has been shown to act as anti-angiogenic factor [Kanda et al., 2002; Kisker et al., 2002]. However, the effect of DBP–maf on the survival of macrophages and the underlying signaling pathways remains unknown. Therefore, we investigated the effect of DBP–maf on survival of macrophages. In this article, we studied the effect of DBP–maf on caspase activity, apoptosis, and cell-cycle distribution in macrophages. We also examined the contribution of the MAPK pathway on activation of macrophages by DBP–maf. Our studies demonstrate for the first time that DBP–maf induces apoptosis in macrophages and that the p38 MAPK pathway plays a pivotal role in this process.

MATERIALS AND METHODS

RPMI cell culture media was purchased from Life Technologies, Inc., (Grand Island, NY); FBS was from Hyclone Laboratories (Logan, UT); 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR); BrdU assay kit for cell proliferation, Sialidase, α-mannosidase, and β-galactosidase were from Roche Molecular Biochemicals (Indianapolis IN); RNase A and CNBr-activated Sepharose 4B were from Sigma Chemical Company (St. Louis, MO); secondary antibody coupled to HRP and Hybond nylon membrane were from Amersham Pharmacia Biotech (Piscataway, NJ), the antibodies, anti-pp38, anti-pJNK1/2, anti-pERK1/2, anti-p38, anti-JNK1/2, and anti-ERK1/2 were from Upstate Biotechnology, Inc. (Lake Placid, NY), and Superoxide dismutase PD98059 and SB202190 were from Calbiochem (San Diego, CA).

RAW 264.7 murine macrophage cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin as recommended by the supplier. Cell culture medium was changed every 3–4 days. The cells were subcultured when 80% confluent.

Preparation of DBP–maf

DBP was purified from pooled human serum by 25-hydroxyvitamin D-affinity chromatography according to the procedure previously described by us [Swamy et al., 1995]. DBP–maf was prepared from DBP by treatment with immobilized sialidase, α-mannosidase, and β-galactosidase according to the reported procedure with suitable modifications [Yamamoto and Kumashiro, 1993]. Briefly, sialidase, α-mannosidase, and β-galactosidase (10 U each) were mixed with 3.0 g of CNBr activated Sepharose (prewashed with 1 mM HCl) in coupling buffer (0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl) at 25°C in an end-to-end shaker

for 4–6 h. The excess reactive sites (on CNBr activated Sepharose) were blocked by incubation with 0.2 M glycine in coupling buffer. The Sepharose immobilized enzymes were washed with coupling buffer to remove free proteins and glycine, followed by 0.1 M acetate buffer (pH 4) containing 0.5 M NaCl. The immobilized enzymes were stored at 4°C after a final wash with coupling buffer until used.

DBP (5.0 mg) was incubated with a mixture of immobilized sialidase, α-mannosidase, and β-galactosidase (5 U of activity each) in PBS–Mg (10 mM sodium phosphate buffer, pH 5.5, 0.9% sodium chloride, and 1 mM $MgSO_4$) at 37°C in an end-to-end shaker for 4 h. The gel was sedimented by centrifugation at 600*g* for 5 min at 4°C. The supernatant (containing DBP–maf) was collected and filter-sterilized using a 0.22-micron filter. Protein estimations were carried out using Bradford's protein assay reagent from BioRad (Hercules, CA) following manufacturer's suggested procedures.

Superoxide Assay

Superoxide production was assessed by monitoring the oxidative burst activity using reduced fluorescein (DCFH-DA) as described by Wan et al. [1993]. Briefly, 50,000 RAW 264.7 cells per well in 24-well plates were treated with DBP or DBP–maf (1 μg/ml/well) in ringer buffer containing 1 mg/ml of ovalbumin for 3 h at 37°C (n = 6). DCFH-DA was added to each well to a final concentration of 2 mM and incubated further for 1 h at 37°C. The fluorescence in the cell free supernatant was determined using Fluorescence-Spectrophotometer (Hitachi F2000, Hitachi High Technologies America, San Jose, CA), excitation at 485 nm and emission at 530 nm. Superoxide dismutase (1 U) was added to control wells to quench superoxide in order to determine the specificity of conversion of reduced fluorescein by superoxide.

DNA Fragmentation Analysis

Apoptosis induced by treatment of RAW 264.7 cells with DBP–maf was studied by DNA fragmentation analysis. The cells (3×10^6) were treated with 15 and 50 μg of DBP or DBP–maf for 72 h. The cells were lysed in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 200 mM NaCl, 0.4% Triton X-100, and 0.1 mg/ml proteinase K) for 20 min at room temperature, followed by a 30 min incubation with 0.1 mg/ml RNase A at 50°C. The lysates were extracted with phenol chloroform and precipitated with cold ethanol. The precipitate was washed with 70% ethanol and dissolved in TE buffer (pH 8.0). DNA was analyzed using 1.5% agarose gel in the presence of 0.5 μg/ml ethidium bromide along side standard DNA size ladder.

Cell Cycle Analysis

Cell cycle analysis and quantification of apoptosis were carried out by flow cytometry. Briefly, the cells were cultured in 6-well plates and treated with various amounts of DBP or DBP–maf in the presence or absence of p38 inhibitor, SB202190 (5 μM) or MEK inhibitor, PD98059 (25 μM) for 48 h. The floating and adherent cells were collected, washed with PBS, fixed and permeabilized with ice-cold 70% ethanol for 30 min followed by incubation with 50 μg/ml propidium iodide and 100 μg/ml RNase for 30 min at 37°C in the dark. Data acquisition and analysis were performed on a FACScan flow cytometer with the accompanying CellQuest software (BD Biosciences, Mountain View, CA). Ten thousand events were analyzed for each sample. Appropriate gating was used to select the single cell population of RAW 264.7 cells. The same gate was used on all samples, ensuring that the measurements were made on a standardized cell population. Determination of the cell cycle distribution was performed twice. The percentage of apoptotic cells was calculated from three independent experiments.

Caspase Activity

The role of caspases in the induction of apoptosis by DBP–maf in RAW 264.7 macrophage cells was investigated by determining the activity of caspase-3, -8, and -9, using Apotarget Caspase-3/CPP32 kit (BioSource International, Inc., Camarillo, CA) following the manufacturer's suggested procedures. Briefly, RAW cells (3×10^6) grown in T25 flasks were treated with 15 μg/ml DBP–maf for 72 h. The cells were lysed with lysis buffer and 100 μg of protein was incubated with 200 μM respective substrates for caspase-3, -8, and -9 for 2 h at 37°C. The absorbance was read at 405 nm. The enzyme activities were expressed as absorbance at 405 nm.

Immunoblotting

The changes in the levels of p38, pp38, JNK1/2, pJNK1/2, ERK1/2, and pERK1/2 in RAW 264.7 cells upon DBP–maf treatment were determined by immunoblotting using specific

antibodies. Cells (5×10^6) growing in mid log phase were treated with different concentrations of DBP–maf (1 ng/ml, 100 ng/ml, 1 μg/ml, and 15 μg/ml) for 24 h. The cells were washed with PBS and lysed with lysis buffer (25 mM HEPES, pH 7.6, 0.1% Triton X-100, 300 mM NaCl, 20 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 2 mM DTT, 0.2 mM Na_3VO_4, 0.2 mM phenylmethyl-sulfonyl fluoride, 4 μg/ml aprotinin, and 2 μg/ml leupeptin). Protein concentrations were determined by using Bradford's protein assay reagent. Protein samples (100 μg) were subjected to SDS–PAGE (12% or 15%) and blotted onto Hybond nylon membrane. The blots were blocked with 5% nonfat dry milk in TBS for 1 h and probed with respective antibodies (p38, pp38, JNK1/2, pJNK1/2, ERK1/2, and pERK1/2) for 3 h at 25°C. After washing, the blots were treated with secondary antibody coupled to HRP for 1 h 25°C. The membranes were washed and developed using enhanced chemiluminescence reagent according to the manufacturer's suggested procedures (ECL System; Amersham Pharmacia Biotech, Piscataway, NJ).

Statistics Analysis

Mean ± standard deviation (SD) were calculated. Mean difference were determined by Student's *t*-test.

RESULTS

DBP–maf Induces Oxidative Burst in RAW 264.7 Cells

Oxygen-dependent mechanisms are known to mediate the anti-microbial and anti-tumor activities of macrophages [Forman and Torres, 2001; Splettstoesser and Schuff-Werner, 2002]. DBP–maf has been shown to increase superoxide production (oxidative burst) in peritoneal macrophages [Yamamoto and Homma, 1991; Yamamoto et al., 1991]. First, we carried out superoxide production assays to establish that RAW 264.7 macrophages respond to DBP–maf by oxidative burst in a similar fashion to primary macrophage cells. We used a sensitive fluorimetric assay developed by Wan et al. [1993] to determine the oxidative burst induced by DBP–maf in RAW 264.7 cells, in which a reduced fluorescein compound is oxidized by the reactive oxygen species. Treatment of RAW 264.7 cells with 1.0 μg of DBP–maf for 3 h resulted in a fivefold increase of superoxide over the untreated control. In contrast, DBP did not show such increase indicating that the observed effect was specific to DBP–maf (Fig. 1). Addition of superoxide dismutase (to specifically quench superoxide) to the culture media resulted in abolition of production of the fluorescent product, which indicates that the increase in fluorescence is due to production of superoxide ions by RAW 264.7 cells. The DBP–maf-induced superoxide production by RAW 264.7 cells was comparable to that of mouse peritoneal macrophages reported by Yamamoto and Homma [1991]. These experiments demonstrate that RAW 264.7 macrophage cells respond to DBP–maf similarly to mouse peritoneal macrophages.

DBP–maf Induces Apoptosis in RAW 264.7 Cells

Programmed cell death (apoptosis) is believed to be an intrinsic process. Several factors are known to induce apoptosis in macrophages [Xaus et al., 2001]. To determine whether DBP–maf induces apoptosis, we treated RAW 264.7 cells with 15 and 50 μg/ml of DBP–maf for 48 h. Genomic DNA was isolated and analyzed for apoptotic DNA fragmentation by agarose gel electrophoresis. DNA fragments characteristic of apoptosis were observed in the case of DBP–maf-treated cells at both concentrations (Fig. 2, Lanes 4 and 5). However, cells treated with DBP

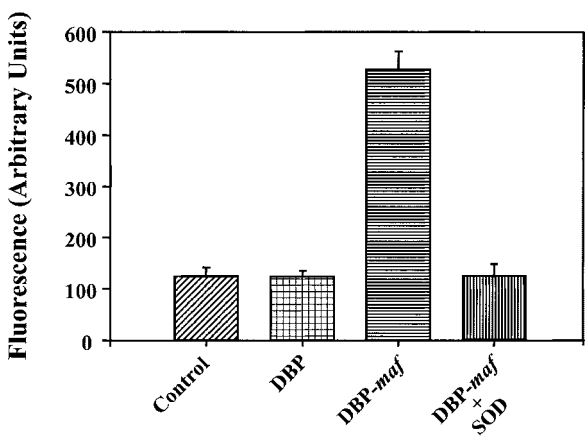

Fig. 1. Oxidative response of RAW 264.7 cells to DBP–maf. Cells grown in RPMI 1640 were treated with DBP or DBP–maf (1 μg/ml/well) in ringer buffer containing 1 mg/ml of ovalbumin for 3 h (n = 6), PBS was used as vehicle control. Oxidative burst activity was determined by adding DCFH-DA to a final concentration to 2 mM and measuring the fluorescence in the cell free supernatant (excitation at 485 nm and emission at 530 nm) after 1 h. Superoxide dismutase (1 U/well) was added to a set of wells to quench the superoxide. Each bar represents the mean of 6-wells ± SD.

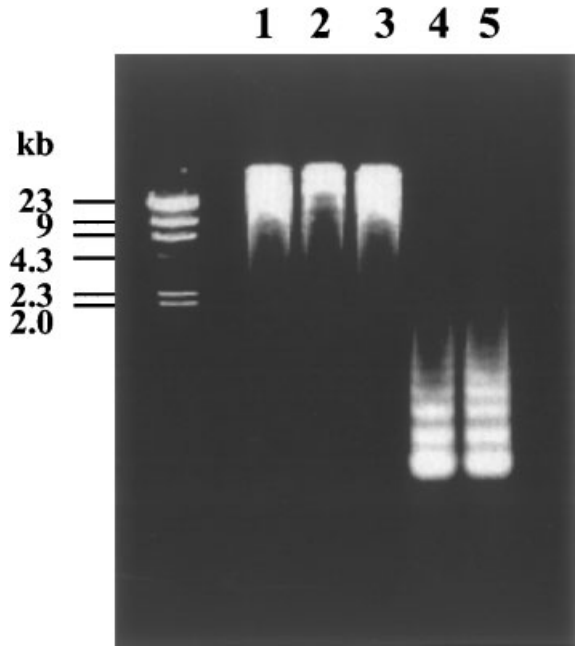

Fig. 2. DBP–maf induced apoptosis in RAW 264.7 macrophage cells. Genomic DNA was isolated from RAW 264.7 cells exposed to 15 and 50 μg of DBP or DBP–maf for 72 h and fractionated with 1.5% agarose gel electrophoresis as described in "Materials and Methods." **Lane 1**: Control (PBS, vehicle treated); **Lane 2**: 15 μg DBP; **Lane 3**: 50 μg DBP; **Lane 4**: 15 μg DBP–maf; and **Lane 5**: 50 μg DBP–maf.

did not show any DNA laddering (Fig. 2, Lanes 2 and 3) indicating that DBP–maf induces cell death by apoptosis in RAW 264.7 cells.

To understand the mechanism of growth suppression of RAW 264.7 cells by DBP–maf and to determine the extent of apoptosis quantitatively, cell cycle analysis was performed. Cells were treated with vehicle control, DBP (100 μg/ml) or DBP–maf (15, 50, and 100 μg/ml) for 48 h and processed for FACS analysis (Fig. 3). In untreated RAW 264.7 cells, only a small fraction of apoptotic cells (2.8% apoptosis) was detected (Fig. 3, Panel a). Treatment with DBP–maf resulted in more extensive apoptosis (Fig. 3, Panel f). Apoptosis was observed in 7.5, 15, and 22% of the cells treated with 15, 50, or 100 μg/ml of DBP–maf, respectively (Fig. 3, Panels c–e) when compared to 2.8% vehicle-treated control cells (Fig. 3, Panel a). DBP did not significantly induce apoptosis (2.6%) and the cell cycle distribution was similar to that of PBS-treated control (Fig. 3, panels a and b). Similarly, DBP did not show any G_0/G_1 block and the cell-cycle profile was similar to that of PBS-treated control (Fig. 3, Panels b and a). DBP–maf treatment increased the proportion of cells in the G_0/G_1-phase of the cell cycle from 52% to 79% at 100 μg/ml concentration and decreased the proportion of S-phase cells from 42% to 18.4%, demonstrating a G_0/G_1 block in cell-cycle progression (Fig. 3, Panels c, d, and e).

The activation of caspase-3, -8, and -9 are specific for apoptosis [Earnshaw et al., 1999]. We determined the activities of caspase-3, -8, and -9 in the DBP–maf-treated cells using specific substrates. There was a sevenfold increase in the activity of caspase-3 when cells were treated with 15 μg/ml DBP–maf. Similarly, there was a fourfold increase in caspase-8 and -9 activities following DBP–maf treatment (Fig. 4). These studies suggest that DBP–maf induces apoptosis of RAW264.7 cells through the caspase pathways.

Role of MAPK in Induction of Apoptosis in RAW 264.7 Cells by DBP–maf

To elucidate the role of MAPK in DBP–maf-induced apoptosis of RAW 264.7 cells, we determined the expression and activity (phosphorylation) of ERK1/2, JNK1/2, and p38 MAPK. RAW 264.7 cells were treated with 1 ng/ml to 15 μg/ml of DBP–maf or DBP for 24 h and the phosphorylated MAPKs were assessed by immunoblotting with specific antibodies recognizing their phosphorylated forms. As shown in Figure 5, DBP–maf induced the phosphorylation of p38 and JNKl/2 and reduced the ERK1/2 phosphorylation. We observed a dose dependent increase in phosphorylation of p38 and JNK1/2 (Fig. 5, Panel a and b). The phosphorylation of p38 reached a maximum at 1 μg/ml dose of DBP–maf (Fig. 5, Panel a, Lanes 2–4) and a further increase in DBP–maf dose to 15 μg/ml resulted in a moderate decrease in phosphorylation (Fig. 5, Panel a, Lane 5). In the case of JNK1/2, phosphorylation increased with increasing doses of DBP–maf (up to 15 μg/ml) (Fig. 5, Panel C, Lanes 2–5). However, phosphorylation of ERK1/2 remained unchanged at 1.0 and 100 ng/ml of DBP–maf (Fig. 5, Panel B, Lanes 2–3) but was inhibited at 1 μg/ml and higher concentrations (Fig. 5, Panel B, Lanes 4–5). DBP failed to significantly inhibit the phosphorylation of p38, JNK1/2, or ERK1/2 under similar conditions (data not shown). To determine the overall expression of the MAPKs, the membranes were stripped and re-probed with antibodies that recognized total forms of p38, JNK1/2, and ERK1/2. As shown in the lower panels of Figure 5, there was no significant change in the total levels of

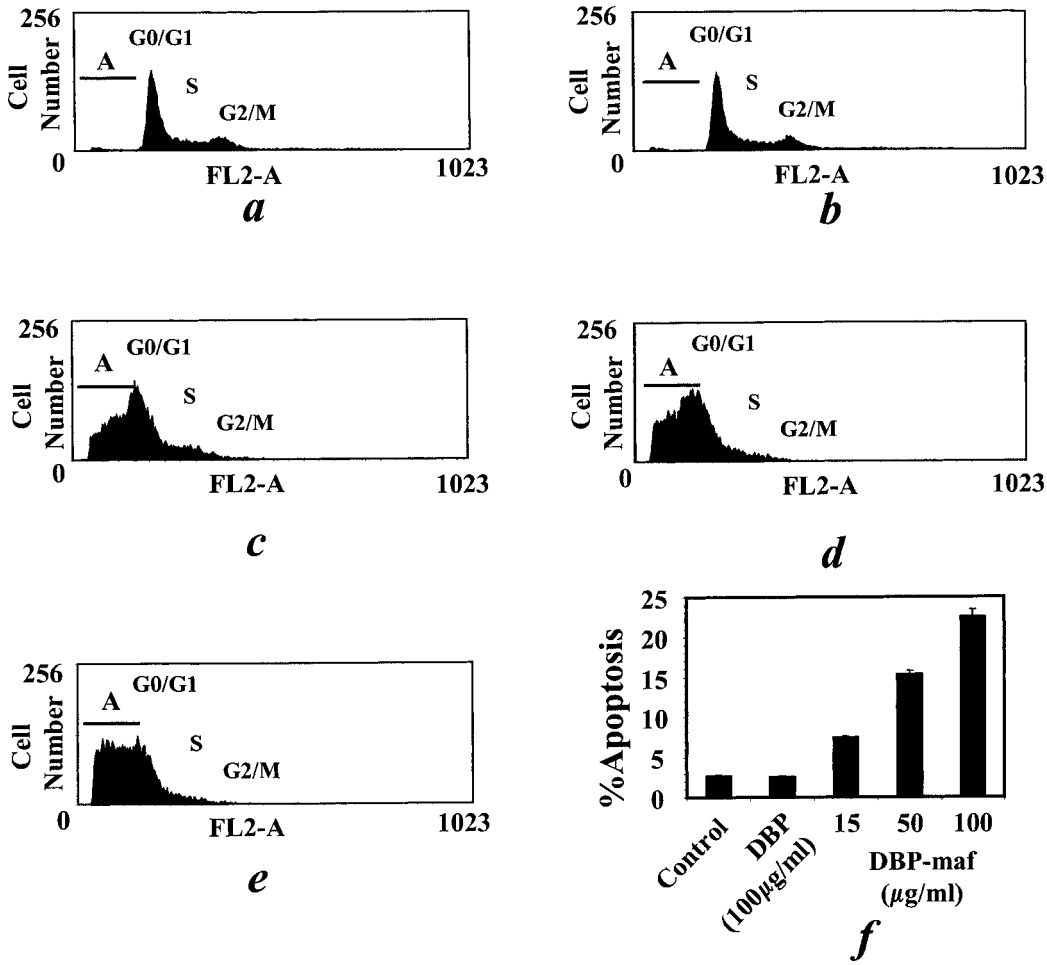

Fig. 3. Cell-cycle distribution following treatment with DBP–maf. RAW 264.7 macrophages were incubated with PBS (vehicle control), DBP (100 μg), or DBP–maf (15, 50, and 100 μg) for 48 h, harvested, stained with propidium iodide, and analyzed by flow cytometry. Cell-cycle distribution was determined as described in the "Materials and Methods." Typical examples of original data are shown. FL2A is orange-red fluorescence 2-area due to propidium iodide staining of DNA. **Panel a**: Vehicle treated control; **Panel b**: DBP (100 μg/ml); **Panel c**: DBP–maf (15 μg/ml); **Panel d**: DBP–maf (50 μg/ml); and **Panel e**: DBP–maf (100 μg/ml). Bar represents sub-G_0/G_1 phase. **Panel f**: The percentage of apoptosis (area under sub-G_1 phase) was determined using CellQuest software during cell cycle analysis as described earlier in control, DBP, and DBP–maf-treated cells. The percentage of apoptotic cells was calculated from three independent experiments.

these proteins indicating that DBP–maf specifically alters the phosphorylation but not the expression of MAPKs. These results suggested that p38 and JNK1/2 MAPK pathways could mediate induction of apoptosis by DBP–maf.

MAP Kinase Pathway Inhibitors Block DBP–maf-Induced Cell Cycle Arrest and Apoptosis

To further assess the role of DBP–maf in activating MAPKs in RAW 264.7 cells, we used p38- and MEK1-specific inhibitors. Specific inhibitors, such as SB202190, block the p38 MAPK pathway without affecting the JNK or ERK pathways. Similarly, PD98059 selectively blocks MEK1, the MAP-kinase kinase responsible for activation of ERK, without affecting the JNK and p38 pathways [Cuenda et al., 1995; Cuvillier et al., 1996; Lee and Young, 1996; Ichijo et al., 1997]. RAW 264.7 cells were incubated with 50 μg/ml of DBP–maf in the presence or absence of SB202190 or PD0980S9 for 48 h and cell cycle progression was analyzed by FACS analysis. Apoptosis was calculated by measuring the area under the curve of the sub-G_1 peak (Fig. 6). Apoptosis induced by DBP–maf treatment (24.2%) was reversed by SB202190 (2%). These studies suggested that p38 plays a critical role in DBP–maf-induced apoptosis in RAW 264.7 macrophages. In

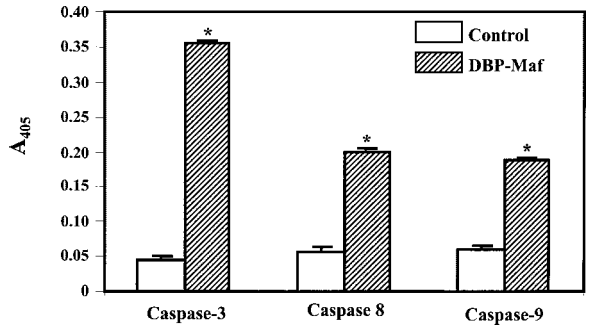

Fig. 4. The activation of caspases in DBP–maf-stimulated RAW cells. RAW 264.7 macrophages (3×10^6 cells) were treated vehicle control (PBS) or 15 μg/ml DBP–maf for 72 h. After lysis, 100 μg of protein was incubated with 200 μM respective substrates for caspase-3, -8, and -9 for 2 h at 37°C. The absorbance was read at 405 nm. The enzyme activities were expressed as absorbance at 405 nm. Values are the mean ± SD of triplicates and asterisks (*) represents a *P* value of less than 0.005.

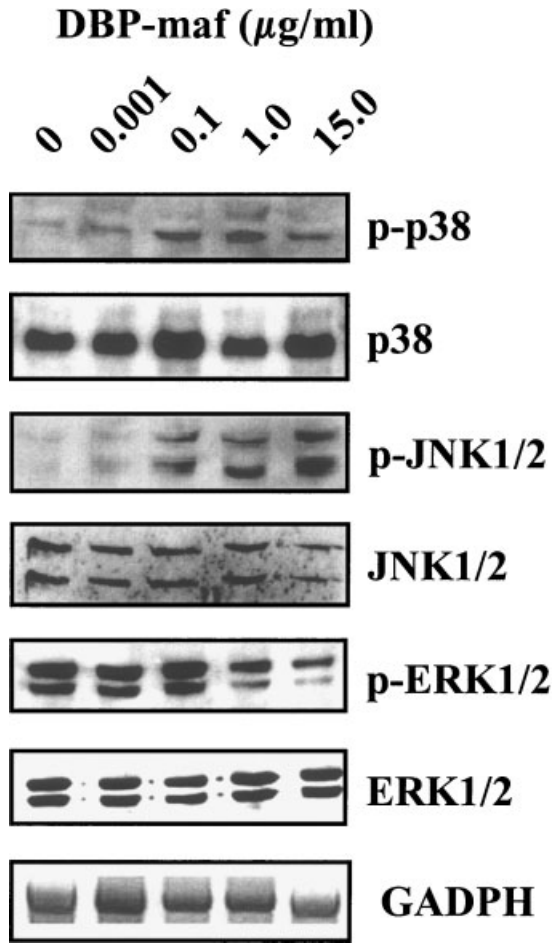

Fig. 5. MAP kinase activation by DBP–maf. RAW 264.7 macrophages (5×10^6 cells) were treated with DBP–maf (1 ng/ml, 100 ng/ml, 1μg/ml, and 15 μg/ml) or PBS (vehicle control) for 24 h. The cells were lysed, protein was electrophoresed and immunoblotted using appropriate antibodies that recognize phosphorylated forms of p38, JNK1/2, and ERK1/2 as described in ''Materials and Methods.'' The blots were stripped and probed with p38, JNK1/2, and ERK1/2 antibodies that recognize phosphorylated and unphosphorylated forms. GADPH was used as a control.

contrast, the addition of PD098059, a MEK1-specific inhibitor protected the cells from apoptosis only marginally. We observed 20.3% apoptosis in the presence of PD098059 as compared to 24.2% in the absence upon DBP–maf treatment. PD098059 or SB202190 in the absence of DBP–maf exerted no apoptotic effects. SB202190 but not PD098059 blocked DBP–maf-induced apoptosis suggesting that the p38 MAPK but not the ERK and JNK MAPK pathways is involved in induction of apoptosis by the DBP–maf. Taken together, these findings clearly indicate that p38-MAPK phosphorylation is required for DBP–maf-induced apoptosis of RAW 264.7 cells.

DISCUSSION

Macrophages play an important role in a series of pathogenic and physiological processes, such as inflammation, infection, anti-tumor, and immune responses and are modulated by a wide variety of stimulatory or suppressive signals [Paulnock, 1994]. Activation by a number of macrophage-activating factors facilitates the action of macrophages [Fedorko, 1999]. Macrophages secrete and respond to a wide range of inflammatory mediators, thereby playing a central role in acute and chronic inflammation. DBP–maf is a newly discovered factor which has been shown to stimulate macrophages to produce superoxide. DBP circulates at a high concentration (200–550 μg/ml) [Cooke and Haddad, 1989] and part or all of the circulating DBP can be potentially converted to DBP–maf at the locus of inflammation. However, not much is known about the effect of DBP–maf on macrophages and the underlying cellular signaling pathways.

In earlier studies, Yamamoto et al. demonstrated that the treatment of peritoneal macrophages with sub-nano-gram quantities of DBP–maf resulted in 4–5-fold increase in superoxide production. Similarly, administration of sub-nano-gram quantities of DBP–maf to mice/rats resulted in increased production of superoxide by peritoneal macrophages [Yamamoto and Homma, 1991, Yamamoto et al., 1991]. Since RAW 264.7 cells recapitulate the behavior of

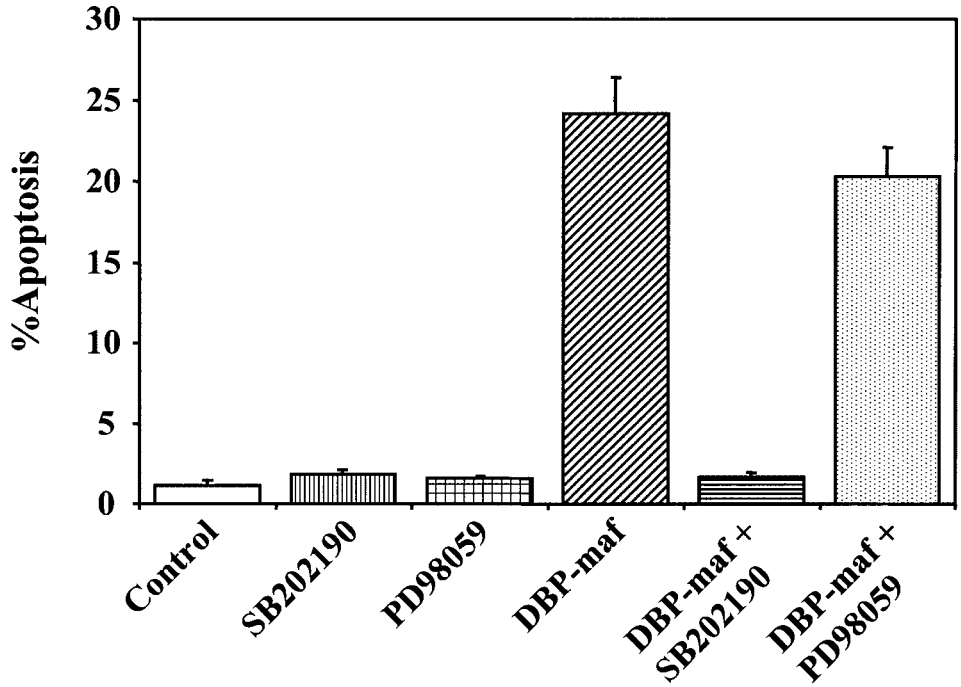

Fig. 6. Inhibition of DBP–maf-induced apoptosis by p38 and MEK inhibitors. RAW cells were treated with 15 μg/ml DBP–maf in the presence or absence of p38 inhibitor, SB202190 (5 μM) or MEK1 inhibitor, PD98059 (25 μM) for 48 h. The cells were harvested, stained with propidium iodide, and analyzed by flow cytometry. Cell-cycle distribution and the percentage of apoptosis (area under sub-G_1 phase) was determined using CellQuest software during cell cycle analysis as described earlier.

peripheral macrophage cells and have been widely used to model events encompassing macrophage activation [Chen et al., 1996], we used this cell line to assess the potential cellular effects of DBP–maf. Similar to primary macrophage cells, RAW 264.7 cells also responded to DBP–maf treatment; increase in the superoxide production was similar to that of the peritoneal macrophages indicating that RAW 264.7 macrophages respond to DBP–maf similar to mouse peritoneal macrophages. Superoxide disumutase (SOD) is used to identify superoxide related events in macrophages and other cells [Koedel and Pfister, 1999]. Addition of SOD to the DBP–maf-treated cells blocked the DBP–maf-induced superoxide production indicating that observed effect is mediated by superoxide.

Macrophages play a critical role during the immune response. Like other cells of the immune system, macrophages are produced in large numbers and most of them die through apoptosis. Apoptosis is known to play an important role in immune system during pathological conditions at the sites of inflammation by eliminating activated macrophages when there is no longer a need for them [Munn et al., 1995; Rathmell and Thompson, 1999]. DBP–maf induced apoptosis in RAW 264.7 in a dose dependent manner. Upon treatment with DBP–maf for 48 h, the cells underwent apoptosis displaying typical apoptotic DNA fragmentation pattern. Cell cycle analysis revealed a G_0/G_1 block with increased apoptosis. Our studies suggest that DBP–maf may play a positive role during inflammation by inducing apoptosis of activated macrophages at the site of inflammation.

Recent advances in the study of apoptosis has revealed that apoptosis involves the activation of caspases in two steps. In the first step, a so-called "initiator" caspase (either caspase-8 or caspase-9) is activated and this caspase, in a second step, activates the "effector" caspase, caspase-3 (and other effector caspases) by limited proteolysis [Earnshaw et al., 1999]. DBP–maf treatment increased caspase-3, -8, and -9 activities with associated apoptosis suggesting that DBP–maf-induced apoptosis is mediated by caspase activation. Activation of macrophages associated with apoptosis has been observed with other macrophage activating factors like TNF-α and IFN-γ [Dellacasagrande et al., 2002].

ERK1/2, JNK1/2, and p38MAPK play a vital role in macrophages activation and apoptosis [Franklin and McCubrey, 2000]. MAPKs are important mediators involved in the intracellular network of interacting proteins that transduce extracellular signals to intracellular responses [Su and Karin, 1996]. DBP–maf decreased the phosphorylation of ERK1/2. There was a concomitant increase in the phosphorylation of P38 and JNK1/2 which was associated with apoptosis of cells. Further proof of involvement of MAPKs was obtained by using MAPK inhibitors. p38-specific inhibitor, SB202190, protected the cells from DBP–maf-induced apoptosis, while MEK-specific inhibitor, PD098059 showed only marginal protection against such apoptosis clearly showing that p38 plays a decisive role in induction of apoptosis.

In summary, this work demonstrates for the first time, that DBP–maf induces apoptosis in cultured RAW 264.7 murine macrophages. DBP–maf treatment results in increased caspase-3, -8, and -9 activities and apoptosis. DBP–maf activates p38 and JNK1/2 MAPKs. On the other hand, ERK1/2 activation is inhibited by DBP–maf. Inhibition of MAPK pathways using specific inhibitors suggests a clear role for p38 MAPK in DBP–maf-induced apoptosis of macrophages. Our studies provide a new insight into the potential contribution of ERK1/2, p38, and JNK1/2 MAPK pathways to the DBP–maf-mediated apoptosis of macrophages.

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