NF-κB-independent Induction of Endothelial Cell Apoptosis by the Vascular Disrupting Agent DMXAA

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Abstract. *Background: DMXAA (5,6-dimethylxanthenone-4-acetic acid; AS1404), a vascular disrupting agent currently in clinical trials, induces tumour endothelial cell apoptosis in vivo in mice and in cancer patients. DMXAA activates NF-κB in many different cell types. In this study, whether DMXAA-induced endothelial cell apoptosis was NF-κB dependent was determined. Materials and Methods: HUVEC endothelial and T24 endothelial-like cells were treated with DMXAA and apoptosis was measured by terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL). NF-κB activation was measured by electrophoretic mobility shift assays (EMSA). T24 cells were transfected with IκBαM, a mutant form of the IκBα gene which cannot be phosphorylated and degraded, hence preventing NF-κB expression. Results: No NF-κB up-regulation was detected in apoptotic HUVEC treated with DMXAA. The IκBαM-transfected T24 cells showed similar apoptotic responses to those of parental cells. Conclusion: The DMXAA-induced apoptosis is neither mediated by, nor inhibited by, the expression of the NF-κB pathway.*

Abbreviations: DMXAA, 5,6-dimethylxanthenone-4-acetic acid; TNF, tumour necrosis factor; HUVEC, human umbilicalcord vein endothelial cells; LPS, lipopolysaccharide; MTT, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; PNPGB phenylmethylsulphonyl fluoride; PMSF, phenylmethylsulphonyl fluoride.

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Vascular disrupting agents are considered to have a number of advantages over conventional chemotherapies for the treatment of cancer as they target a genetically stable population of endothelial cells, which are less prone to develop resistance to therapy compared to tumour cells. DMXAA (5,6-dimethylxanthenone-4-acetic acid), developed at the Auckland Cancer Society Research Centre, New Zealand, is one of several such drugs that have reached advanced stages of clinical evaluation. Data from Phase I trials showed that DMXAA reduced blood flow to tumours in patients (2) and following promising Phase II combination trials for the treatment of lung cancers, the drug is now scheduled to enter Phase III evaluation. As part of our investigation for its anti-vascular action, it was shown that DMXAA caused apoptosis of vascular endothelial cells in murine Colon 38 tumours in mice as early as 30 min following administration, when damage was not yet observable on the tumour cells. Apoptosis of the tumour vascular endothelium increased with time as did the appearance of necrotic areas within the tumour (3). As determined by transferase biotin-dUTP nick end labelling (TUNEL)-staining, apoptosis induction was selective to tumour vascular endothelium and no TUNEL-staining was observed in the vasculature in the mouse spleen, heart, brain or liver after DMXAA treatment (4). A strong correlation was obtained between inhibition of tumour blood flow and the percentage of apoptotic vessels in the Colon 38 tumour (4), indicating that apoptosis induction played a contributing or causative role in the anti-vascular action of DMXAA. Apoptotic endothelial cells were also observed in breast cancer biopsies taken from a patient, 3 and 24 h, post infusion of DMXAA (3), consistent with the involvement of endothelial cell apoptosis in the reduction of tumour blood flow observed in patients in the Phase I trials (2, 5).

Another key component of the activity of DMXAA in preclinical models is the activation of intra-tumoral cytokine biosynthesis (6, 7), which can augment and extend the initial

direct effects on the vasculature (8) leading to irreversible and profound blood flow collapse, tumour ischemia and necrosis (9, 10). Recent studies showed that the inhibition of NF-κB using parthenolide or salicylate resulted in loss of DMXAA-induced production of tumour necrosis factor (TNF) and IFN-γ production *in vitro*, indicating a role of NF-κB (11). DMXAA has been shown to activate NF-κB *in vivo* in tumours, as well as in a number of murine and human tumour lines in culture (6, 12, 13). In the murine ANA-1 macrophage cell line, activation of NF-κB appeared to be associated with up-regulation of cytokine gene transcription (14).

NF-κB is also involved in regulating apoptosis (15) and its role can be pro- or anti-apoptotic depending on context, type of cell, and type of stimulus and may likely involve other transcription factors, such as AP-1 (16). While the involvement of NF-κB in blocking apoptosis is predominant in the literature (17), there are also reports of the pro-apoptotic aspect of NF-κB. Both Fas-ligand and its receptor Fas, a member of the TNF death receptor superfamily, have NF-κB binding sites at their promoters and are induced by NF-κB (18-20). NF-κB was also found to be important for p53-mediated apoptosis (21). There have been additional studies on the role of NF-κB in promoting apoptosis in endothelial cells. Bovine and porcine aortic endothelial cells were shown to activate NF-κB upon stimulation with ATP or ADP, and underwent apoptosis (22). Inhibition of NF-κB with various antioxidants suppressed high glucose-induced apoptosis in human umbilical cord vein endothelial cells (HUVEC) (23). NF-κB was pro-apoptotic in the induction of apoptosis by the anti-cancer drug doxorubicin (24), as suppressed NF-κB activation in doxorubicin-treated bovine aortic endothelial cells decreased caspase-3 activity and TUNEL-staining (24).

In this study, whether NF-κB was involved in DMXAA-induced endothelial cell apoptosis was investigated. If the induction of endothelial cell apoptosis could also be shown to involve NF-κB, then both its anti-vascular and cytokine inducing effects of DMXAA could be attributed to a common signalling pathway. The relationship between NF-κB activation and induction of apoptosis in HUVEC and the human T24 (also known as ECV304) endothelial-like cell line was investigated.

Materials and Methods

Drugs and reagents. The sodium salt of DMXAA, synthesized at the Auckland Cancer Society Research Centre, was dissolved in culture medium immediately before use and the pH adjusted to 7.5. Human recombinant TNF was obtained from PharMingen (San Jose, CA, USA) and lipopolysaccharide (LPS), serotype 055:B5 was from Sigma (St. Louis, MO, USA). Anti-IκBα was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. T24 was obtained from American Type Culture Collection (Bethesda, MD, USA) and cultured in α-modified minimal essential medium (Gibco BRL Life Technologies, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml ampicillin and 100 μg/ml streptomycin. HUVEC were isolated as previously described (25) from umbilical cords obtained from donors with informed consent. HUVEC were cultured in M199 medium supplemented with 10% (v/v) FCS, 1 U/ml ampicillin, 1 μg/ml streptomycin and 2 mM L-glutamine in gelatin-coated tissue culture flasks. HUVEC between passages 2-3 were used for experiments and all experiments had approval from the Regional Ethics Committee. All cultures were maintained at 37˚C under humidified atmosphere of 5% CO_2.

Generation of IκBα and IκBαM stably-transfected T24 cell lines. T24 cells were transfected with vectors containing either the wild-type IκBα or the dominant negative IκBαM mutant gene (serine to alanine mutation at residues 32 and 36) driven by a constitutive CMV promoter (Clontech Inc., Palo Alto, CA, USA) using Lipofectamine 2000 reagent (Gibco BRL) according to the manufacturer's instructions. Following transfection, the cells were incubated with fresh M199 medium supplemented with 0.8 mg/ml G418 sulphate (Gibco BRL). The cells were harvested after 2 weeks and were then plated into 96-well plates at 6 cells/well and maintained for an additional 2 weeks. Wells containing a single colony were selected for development into stably transfected clones.

Cell growth and cytotoxicity determinations. Cells were initially seeded at 10^4 per plate in duplicate plates, harvested every two days for 10 days and counted. Cell viability was assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (26). Wild-type and transfected T24 cells ($1.5x10^4$ cells/well) were seeded in triplicates in 96-well flat-bottom plates and incubated at 37˚C for 1 h. DMXAA was added to the cells from a separate plate at 2-fold dilution to final concentrations of 10 to 1000 μg/ml. After incubation at 37˚C for 24 h, 10 μl MTT (5 mg/ml) were added per well and the cultures further incubated for 1 h. Once the formazan crystals were observed, the culture media was discarded and 100 μl DMSO were added. The absorbance was then measured at 550 nm with an automated microplate reader ELx808 (Bio-Tek Instruments Inc., Winooski, VT, USA).

Apoptosis assay. Apoptotic cells were detected using the TUNEL assay for identification of double-stranded DNA breaks using an *in situ* cell death detection kit (Roche Diagnostic GmbH, Mannheim, Germany) according to manufacturer's instructions. Cytospots of cells on poly-L-lysine-coated slides were fixed in 4% paraformaldehyde solution for 1 h at room temperature, washed in 0.1 M PBS (3x10 min) and then treated with permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 3 min on ice, followed by PBS washes (3x10 min). Strand breaks were labelled with fluoresceinated dUTP and visualised following reaction with phosphatase-conjugated antibody to fluorescein and Vector® Red alkaline phosphatase substrate solution (Vector Laboratories Inc., Burlingame, CA, USA). A minimum of 500 cells was counted for each slide and the results were expressed as percentage of apoptotic cells over total number of cells counted. Statistical analysis was performed using the Student's *t*-test compared to untreated controls.

Western blotting. Cells ($2x10^6$) were harvested, washed and resuspended in PBS. An equal volume of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol

blue) was added and the samples vortexed thoroughly. Phenylmethylsulphonyl fluoride (PMSF) was added to a final concentration of 1 mM and the DNA in the samples sheared using a syringe. Protein concentration was determined with the BCA reagent (BioRad, Hercules, CA, USA) at 550 nm using an automated microplate reader ELx808 (Bio-Tek Instruments Inc.). Samples (30 μg protein in 5% 2-mercaptoethanol) were heated at 98˚C for 4 min, loaded onto a 12% polyacrylamide gel and subjected to electrophoresis at 120 V for 1 h 15 min in 1x electrophoresis buffer (200 mM glycine, 15 mM Tris, 3.5 mM SDS). The separated proteins were then transferred to nitrocellulose membrane (BioRad) using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) at 100 V for 1 h. The nitrocellulose membrane was blocked with the blocking buffer (0.05% Tween in PBS, 5% (w/v) non-fat milk powder) for 1 h at room temperature and washed with 0.05% Tween in PBS (3x5 min). The primary antibody solution (1:1,000 dilution) of anti-IκBα mouse monoclonal antibody was allowed to incubate with the membrane overnight at 4˚C. The following day, the antibody was washed off with 0.05% Tween in PBS (3x5 min). Incubation with the secondary horse-radish peroxidase-conjugated goat anti-mouse antibody was carried out for 1 h at room temperature, followed by washing with 0.05% Tween in PBS (3x5 min). Chemiluminescence of IκBα protein-antibody complexes were visualised with the SuperSignal West Pico Trial Kit (Pierce Biotechnology, Rockford, IL, USA).

Nuclear protein extraction. Cells ($2.5x10^7$) were incubated with DMXAA, LPS or TNF and harvested after 2 h. Nuclear proteins were prepared, as previously described (6, 14). Cells were lysed in cell lysis buffer (15 mM KCl, 10 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 0.1 mM EDTA, 25 μM dithiothreitol (DTT), 25 μM phenylmethylsulphonyl fluoride (PNPGB) and 0.5% Nonidet P-40) for 30 min. The pellet of nuclei was then incubated in 10 μl nuclei lysis buffer (0.5 M KCl, 25 mM HEPES (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 25 μM PNPGB) on ice for 30 min followed by the addition of 100 μl dialysis buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 25 μM PNPGB). After centrifugation at 20,000 xg for 15 min, the supernatant was collected and the protein concentration was determined with Bradford reagent at 596 nm (27).

Assay for NF-κB activation. NF-κB complexes in the nuclear extracts were determined using the electrophoretic mobility shift assay (EMSA), as previously described (14). Briefly, the oligonucleotide (5'-AGCTTACAAGGGACTTTC-3') containing the NF-κB consensus binding site from the k immunoglobulin enhancer gene (28), annealed to its complementary strand, was radiolabelled using the Klenow fragment of DNA polymerase I (Klenow Fill-In Kit, Stratagene, CA, USA) and [α-^{32}P]dCTP (370 mBq/ml, 10 mCi/ml, Redivue, Amersham) in a fill-in reaction for 5'-protruding ends. DNA binding reactions were carried out in total volume of 15 μl containing 5 μg nuclear protein, 4 μl binding buffer (20 mM KCl, 12 mM HEPES, pH 7.6, 2.5 mM $MgCl_2$, 0.4 mM EDTA, 0.5 mM DTT, 25 μM PNPGB, 12% glycerol) and 1.5 μg poly (dI:dC). The samples were incubated on ice for 10 min before adding ^{32}P-labelled probe (20,000 cpm). Reactions were terminated by the addition of a loading dye (250 mM Tris (pH 7.5), 0.2% bromophenol blue, 0.2% xylene cyanol and 4% glycerol). Samples were loaded onto a 4% polyacrylamide gel and subjected to electrophoresis in 0.25 X TBE buffer (22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA) at 150 V for 2 h. The dried gels were exposed to autoradiography (Kodak Scientific Imaging Film) at –70˚C overnight.

Results

Effect of DMXAA on HUVEC. The cytotoxicity of DMXAA was first measured on HUVEC isolated from umbilical cords obtained from two individual donors. HUVEC were cultured with varying concentrations of DMXAA and cell viability was measured after 24 h using the MTT assay. HUVEC from each donor were tested using quadruplicate cultures and the results were combined in Figure 1A. The concentration of DMXAA which caused 50% loss of cell density (IC_{50}) was determined to be 300 μg/ml. To ascertain if this reduction was due to induction of apoptosis, HUVEC were treated with DMXAA at varying concentrations for 24 h and then processed for TUNEL staining. The percentage of TUNEL-stained cells in each culture was counted and the mean from four independent experiments, each using HUVEC from a different donor, are shown in Figure 1B. Treatment with DMXAA concentrations at or above 300 μg/ml produced a statistically significant increase in apoptotic cells compared to that in untreated controls, with the highest number (30%) of TUNEL-stained cells obtained at 750 μg/ml. The percentage of apoptotic cells at 1,000 μg/ml DMXAA was lower than that obtained at 500 and 750 μg/ml and could be due to complete disintegration of the apoptotic cells and their disappearance from the culture. Whether DMXAA activated NF-κB in HUVEC at concentrations where apoptosis was induced was subsequently investigated. HUVEC were cultured with DMXAA at varying concentrations, and nuclear proteins from the cells were extracted after 2 h and assayed for translocated NF-κB. No activation of NF-κB above that of untreated controls was observed in three independent experiments using HUVEC from different donors. To guard against possible technical problems, all EMSAs included a sample known to contain activated NF-κB (positive control), as well as a sample known not to contain activated NF-κB (negative control). A representative EMSA is shown in Figure 1C, indicating no activated NF-κB in nuclear extracts from the DMXAA-treated HUVEC.

The effect of DMXAA on IκBα and IκBαM stably-transfected T24 cells. The studies using HUVEC indicated that DMXAA-induced apoptosis could occur in the absence of NF-κB activation. Whether the abrogation of IκB function would prevent DMXAA-induced apoptosis was tested by transfecting cells with IκBαM, a mutant form of the IκBα gene that lacks phosphorylation sites at serines 32 and 36 (29). The corresponding protein therefore cannot be phosphorylated or degraded, thus preventing NF-κB activation. The T24 human tumour cell line, known to express endothelial cell functions (30, 31) was utilized and transfected with either wild-type IκBα or IκBαM. The behaviour of clone 4 (IκBα transfectant) or clones 10 and 17 (IκBαM transfectants) was compared with that of non-transfected wild-type cells.

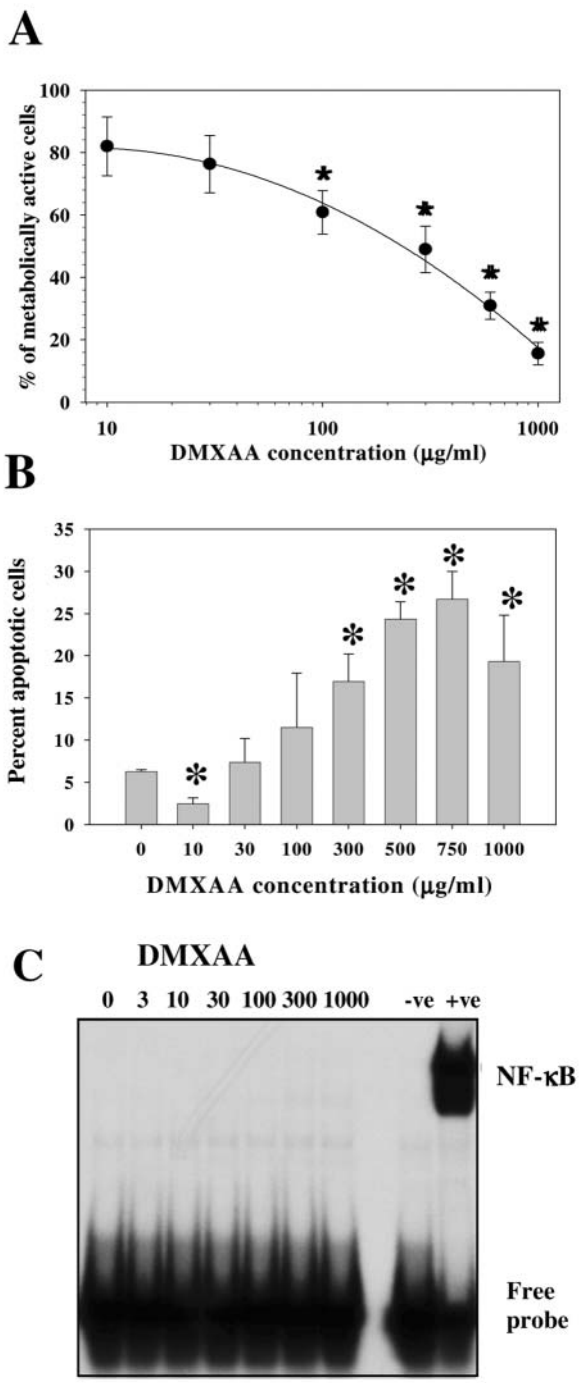

Figure 1. *A) Survival of HUVEC in culture 24 h after treatment with varying concentrations of DMXAA as assessed by MTT assay. *p≤0.05 of treated compared to untreated. B) Percent TUNEL-stained HUVEC in culture 24 h after treatment with DMXAA. *p≤0.05 compared to untreated. C) EMSA showing no activated NF-κB bands in nuclear proteins extracted from HUVEC treated with indicated concentrations of DMXAA for 2 h. Samples known to be positive or negative for activated NF-κB were included as positive and negative controls for the EMSA gel.*

IκBα or IκBαM expression in stable transfectant lines selected for investigation were determined by Western blotting (Figure 2A). Clone 4 expressed similar amounts of cellular IκBα to that of wild-type T24 cells (Figure 2A: I), clone 10 expressed comparable amounts of IκBαM and clone 17 expressed high levels of IκBαM (Figure 2A: II). No morphological differences were observed among any of the clones and the wild-type T24 when viewed under phase contrast microscopy and neither were there any obvious differences in growth rates for any of the clones compared to wild-type T24 (Figure 2B). Sensitivity to DMXAA was determined using the MTT assay after 24-h incubation with varying concentrations of the drug and showed no significant differences between the clones and the wild-type T24 cells (Figure 2C). The IC_{50} of DMXAA for T24 wild-type cells and for all the clones was 650 μg/ml.

The transfected clones were next compared with wild-type T24 cells for NF-κB activation following treatment with DMXAA. LPS and recombinant human TNF, which are known NF-κB activators, were used as positive controls (Figure 3). TNF was the strongest activator of NF-κB in wild-type T24 cells followed by LPS and then DMXAA (Figure 3A). Clone 10 still exhibited a strong NF-κB response to TNF, although the responses to DMXAA at 300 μg/ml and LPS at 100 ng/ml were diminished as compared with that of wild-type cells (Figure 3A). In contrast, clone 17, which expressed high amounts of IκBαM (Figure 2A II), showed no activated NF-κB when treated with DMXAA and LPS and a severely reduced response to TNF (Figure 3B). Thus, the high expression of the mutant IκBαM in clone 17 resulted in marked attenuation of the NF-κB response in those cells, although no obvious effects in growth rates (Figure 2B) or sensitivity to DMXAA-induced cytotoxicity (Figure 2C) was evident.

The transfected clones were then compared with wild-type T24 cells for apoptosis induction following treatment with DMXAA. The cells were treated with DMXAA at the IC_{50} (650 μg/ml) for 24 h and were then stained for apoptotic cells using TUNEL. The number of TUNEL-stained cells per 500 cells was then counted. Three independent experiments were carried out and a representative field of untreated and DMXAA-treated wild-type T24, clone 4, clone 10, and clone 17 cells from one experiment is shown in Figure 4. The number of apoptotic cells in untreated samples in Figure 4 was below 5% and treatment with DMXAA resulted in a 5-fold increase in apoptotic cells in wild-type cells, a 6.4-fold increase in clone 4, 8.7- and 16.4-fold increases in clone 10 and clone 17, respectively. When the results from the three independent experiments were compared, no significant difference ($p=0.4$) in percentage of apoptotic cells was observed between clone 10, the low-expressor (6.0±1.6%) and clone 17, the high-expressor IκBαM-transfected clone (11.6±5.8), despite a clear difference in their ability to activate NF-κB (Figure 3B). There was also no significant

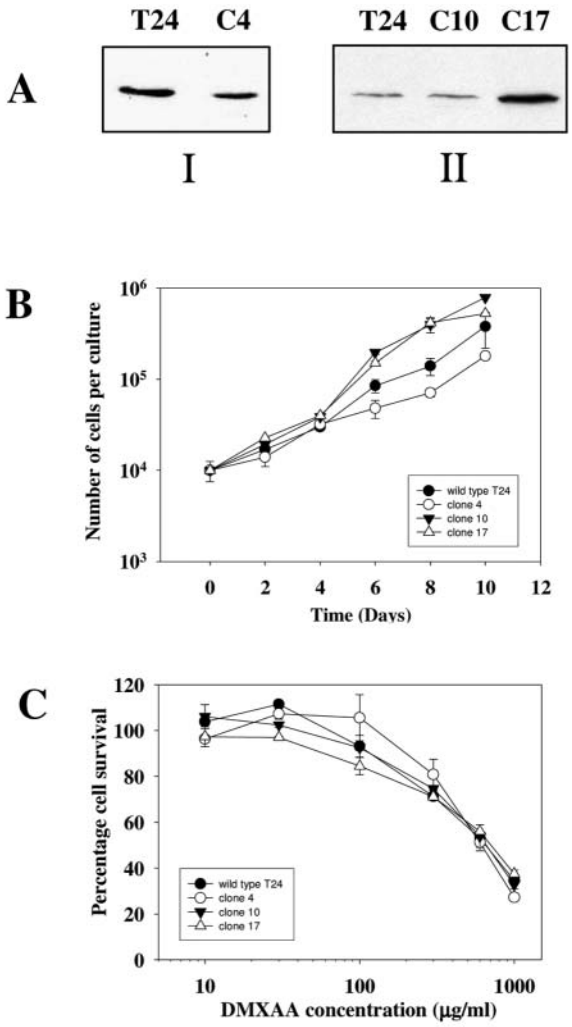

Figure 2. *A) Western blots of IκBα expression in IκBα stably-transfected clone (C4) compared with non-transfected wild-type T24 cells (I) and IκBαM stably-transfected clones (C10, C17) compared with wild-type (II). B) Growth of wild-type T24 cells (filled circles), clone 4 (open circles), clone 10 (filled triangles) and clone 17 (open triangles) measured over 12 days. C) Percentage cell survival of wild-type T24 cells (filled circles), clone 4 (open circles), clone 10 (filled triangles) and clone 17 (open triangles) 24 h after treatment with varying concentrations of DMXAA as determined by MTT assay.*

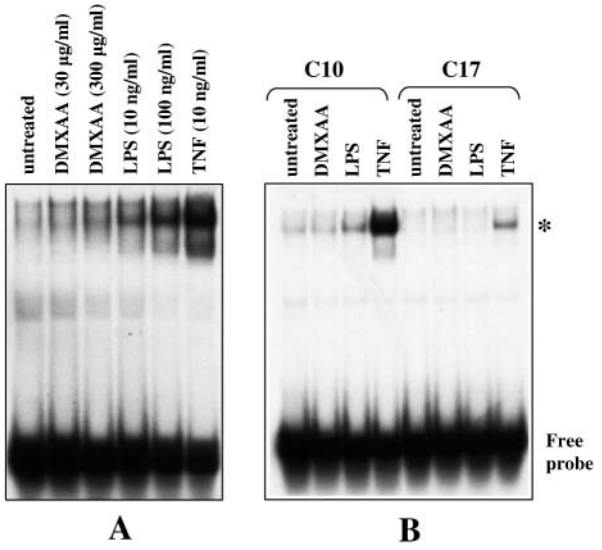

Figure 3. *EMSA of activated NF-κB in nuclear extracts from: (A) wild-type T24 cells without treatment, or treated 2 h with indicated concentrations of DMXAA, LPS or TNF; (B) IκBαM stably-transfected clones C10 and C17, untreated or 2 h following treatment with DMXAA (300 µg/ml), LPS (100 ng/ml) or TNF (10 ng/ml). *denotes NF-κB.*

difference ($p=0.4$) observed in apoptosis induction between clone 4, the IκBα-transfected clone (4.7±0.9) and wild-type T24 cells (3.6±0.9).

Discussion

With DMXAA scheduled to enter Phase III clinical trials, there is considerable interest in the signalling pathway and biochemical receptor for this agent. Unlike the majority of the other vascular-disrupting agents that are also in clinical development, DMXAA does not appear to involve tubulin binding as its prime target of action. Previous studies from this laboratory have implicated an involvement of the NF-κB pathway in the production of cytokines induced with DMXAA (11, 14). Since NF-κB has been documented to be involved in apoptotic events in endothelial cells (15, 32, 33), a possible role of NF-κB in the endothelial cell apoptosis induced by DMXAA *in vitro* was investigated. The results using two different endothelial cell models strongly indicate that DMXAA-induced apoptosis in culture is independent of NF-κB activation. In cultures of primary HUVECs, apoptosis was induced in the absence of any NF-κB activation (Figure 1). Using T24, an endothelial-like tumour cell line formerly known as ECV304, no significant difference was found between growth, cytotoxicity and apoptosis induction in response to DMXAA between wild-type cells and clones with an attenuated NF-κB response (Figure 3) through stable transfection with an inactive form of IκBα (IκBαM). Since IκBαM cannot be activated (29) in these clones, IκBα does not become phosphorylated and degraded (34) to release the NF-κB for translocation into the nucleus to activate gene transcription, including those regulating apoptosis (35, 36). The IκBαM-stably transfected clones provided an attractive system to investigate whether decreased NF-κB activity affected apoptosis induction by DMXAA. The increase in apoptotic cells following DMXAA treatment in IκBαM-transfected clones was not significantly different to that observed in non-transfected wild-type cells, or to that in clones transfected with the gene for the active IκBα.

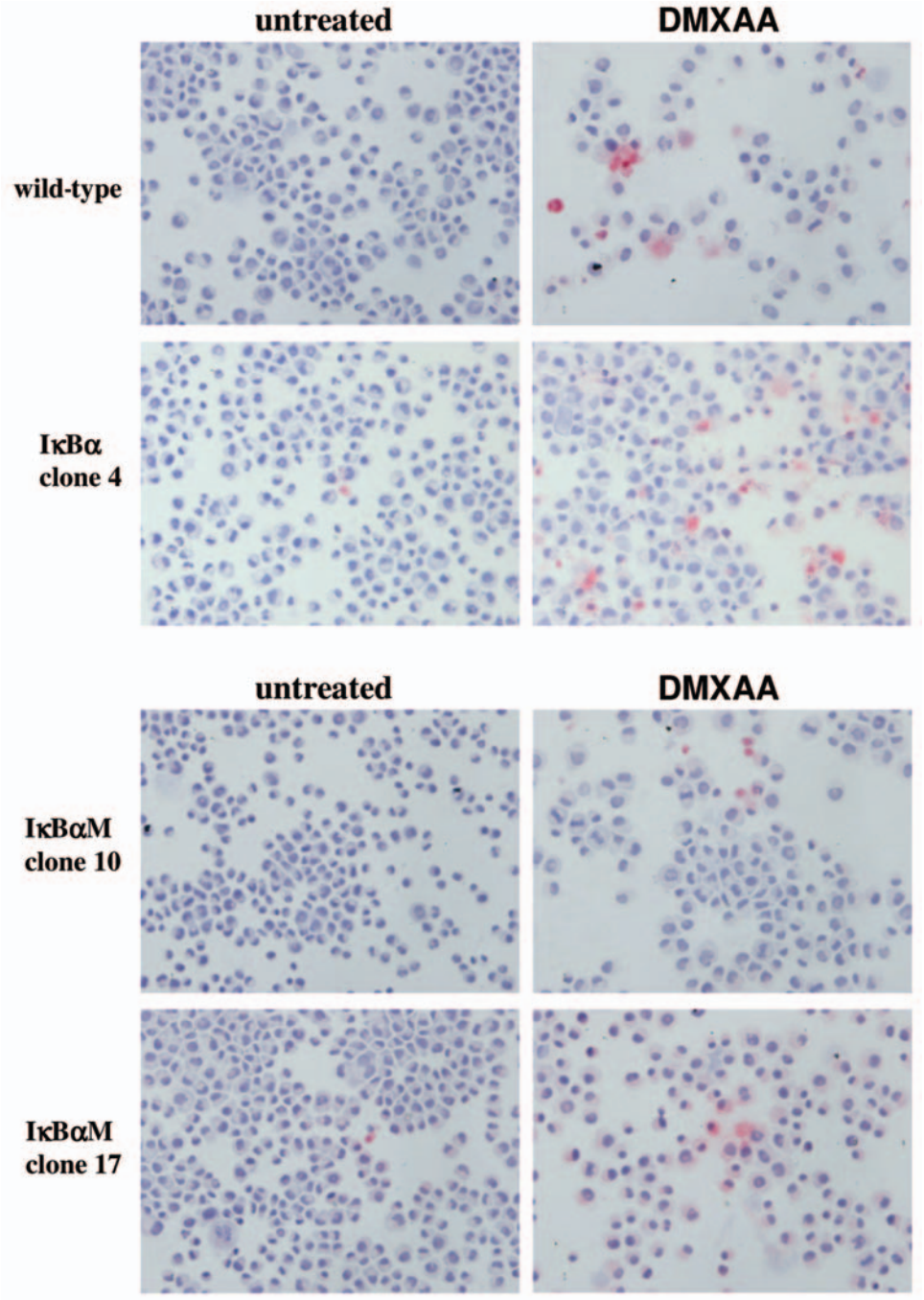

Figure 4. *TUNEL-stained cells (red) in a representative field of untreated (left hand column) or DMXAA-treated (650 μg/ml for 24 h) (right hand column) of wild-type T24 cells or stably-transfected clones 4, 10 and 17.*

Our results suggest that NF-κB is not involved in the induction of apoptosis by DMXAA, prompting the question of which pathway(s) was involved. There are numerous pathways for apoptosis and they eventually converge on the activation of caspases -3, -6 and -7 that lead to further processing of downstream substrates (37). In particular, caspase-3 is central to both death receptor and mitochondria-mediated pathways and its activation results in DNA fragmentation, a classic marker of apoptosis detected using a DNA ladder assay (38-40). DNA fragmentation is mediated by endonucleases, most notably caspase-activated deoxy-ribonuclease/DNA

fragmentation factor (41), which is dependent on caspase-3 for activation by cleavage of its inhibitory subunit (42-45). The TUNEL assay, which stains for DNA strand breaks, was positive in DMXAA-treated HUVEC (Figure 1A), murine endothelial cell lines (3), as well as the T24 human endothelial-like cell line (Figure 4), suggesting endonuclease activity.

Polychlorinated biphenyl-induced apoptosis in human microvascular endothelial cells was shown to be independent of NF-κB, AP-1 and STAT1 and specifically involved CREB (46). DMXAA-induced apoptosis on endothelial cells, as shown in these studies, was also independent of NF-κB. However, there is compelling evidence that cytokine induction by DMXAA does involve NF-κB (11, 14). TNF and IFN-γ production in splenocytes promoted by DMXAA has been found to be inhibited by salicylate and parthenolide (11), both inhibitors of the IKK-β subunit of the IKK complex (47-49) responsible for NF-κB activation. If NF-κB is involved in DMXAA-induced cytokine production, but not in DMXAA-induced apoptosis, it can be concluded that DMXAA must act through multiple pathways. Which pathway utilised may depend on the target cell or cellular context. The resultant action of DMXAA will, thus, be dependent on cell type and may provide the basis for the multiple activities of the drug. Furthermore, there may be different targets for DMXAA. Identification of these various pathways and targets is crucial to our understanding of the action of DMXAA and the optimisation of its clinical potential.

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