Induction of endothelial cell apoptosis by the antivascular agent 5,6-dimethylxanthenone-4-acetic acid

5,6-Dimethylxanthenone-4-acetic acid (DMXAA), synthesised in this laboratory, reduces tumour blood flow, both in mice and in patients on Phase I trial. We used TUNEL (TdT-mediated dUTP nick end labelling) assays to investigate whether apoptosis induction was involved in its antivascular effect. 5,6-Dimethylxanthenone-4-acetic acid induced dose-dependent apoptosis in vitro in HECPP murine endothelial cells in the absence of up-regulation of mRNA for tumour necrosis factor. Selective apoptosis of endothelial cells was detected in vivo in sections of Colon 38 tumours in mice within 30 min of administration of 5,6-Dimethylxanthenone-4-acetic acid (25 mg kg⁻¹). TUNEL staining intensified with time and after 3 h, necrosis of adjacent tumour tissue was observed. Apoptosis of central vessels in splenic white pulp was also detected in tumour-bearing mice but not in mice without tumours. Apoptosis was not observed in liver tissue. No apoptosis was observed with the inactive analogue 8-methylxanthenone-4-acetic acid. Positive TUNEL staining of tumour vascular endothelium was evident in one patient in a Phase I clinical trial, from a breast tumour biopsy taken 3 and 24 h after infusion of 5,6-Dimethylxanthenone-4-acetic acid (3.1 mg m⁻³). Tumour necrosis and the production of tumour necrosis factor were not observed. No apoptotic staining was seen in tumour biopsies taken from two other patients (doses of 3.7 and 4.9 mg m⁻³). We conclude that 5,6-Dimethylxanthenone-4-acetic acid can induce vascular endothelial cell apoptosis in some murine and human tumours. The action is rapid and appears to be independent of tumour necrosis factor induction.

Keywords: DMXAA; apoptosis; antivascular; Colon 38 tumour; endothelial

Materials and Methods

Materials

DMXAA was synthesised in this laboratory (Rewcastle et al, 1991) and dissolved in 5% sodium bicarbonate for intraperitoneal injection into mice (25 mg kg⁻¹) in a volume of 0.01 ml g⁻¹ body weight. DMXAA was dissolved directly in culture medium for experiments. In the clinical phase trial conducted in Auckland, New Zealand, DMXAA was administered as a 20 min intravenous infusion on a 3-weekly schedule, using a pre-formulated solution of 20 mg ml⁻¹ in 0.1 M phosphate buffer at pH 7.7 (Jameson et al, 2000).

Mouse studies

Athymic or normal C57BL/6 mice were from the Animal Laboratories, University of Auckland School of Medicine. All mice were...
maintained under constant temperature and humidity according to institutional ethical guidelines and used between 8–12 weeks of age. All animal experiments have been carried out with ethical committee approval. The ethical guidelines that were followed meet the standards required by the UKCCCR guidelines (Workman et al., 1998). Colon 38 tumour fragments (1 mm³) were implanted subcutaneously in the left flank of anaesthetised (sodium pentobarbitone, 86 mg kg⁻¹) normal C57Bl/6 mice. Tumours were used when they had reached approximately 6 mm in diameter, generally 9–10 days after implantation. Three mice were used for each time point and treatment, and 2–4 cryosections per organ per mouse were immunostained for apoptosis using TUNEL.

Clinical studies

Three patients with superficial tumours (two with chest wall recurrence of breast adenocarcinoma and one with subcutaneous ovarian adenocarcinoma tumour nodules) consented to biopsies. These samples were taken prior to infusion of DMXAA then at various intervals afterwards and were embedded (OCT compound, a mixture of water-soluble glycols and resins, Tissue-Tek®; Sakura Finetech, Torrance, CA, USA) and snap-frozen. Representative cryosections were immunostained for apoptosis using TUNEL.

In vitro studies

The HECPP murine endothelial cell line derived from endothelial cells isolated from murine Peyer’s patches (Bizourne et al., 1993) was maintained in M199 media (Gibco BRL) with antibiotics and 10% foetal calf serum at 37°C under humidified atmosphere of 3% CO₂. Sub-confluent cultures (that had been passaged the previous day) were incubated with DMXAA for the appropriate time. The supernatant containing non-adherent cells was removed and centrifuged at 300 × g for 10 min. Adherent cells were lifted from the dishes by trypsinisation and collected similarly. Combined adherent and supernatant cells, resuspended in culture medium (0.4 ml), were used to prepare cytospin preparations that were assayed for apoptosis.

Apoptosis assay

Apoptosis was determined using the TUNEL assay for identification of double-stranded DNA breaks using an In Situ Cell Death Detection Kit (Boehringer Mannheim) according to manufacturer’s instructions. Tissue and tumour cryosections or cytospots of cells on poly-L-lysine-treated slides were fixed in 4% paraformaldehyde for 30 min at room temperature, washed with PBS (phosphate buffered saline) and then treated with permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Strand breaks were labelled with fluorescein diUTP and visualised following reaction with phosphatase-conjugated antibody to fluorescein and Vector® Black alkaline phosphatase substrate solution (Vector Laboratories, Burlingame, CA, USA). All slides were counter stained using methyl green.

Cryosections of Colon 38 tumour tissues were also stained for endothelial cells to differentiate between apoptosis of the endothelium and apoptosis of tumour cells. Sections were first processed for apoptosis as described and then immunostained for endothelial cells using a rat anti-mouse CD-31 antibody (MEC 13.3, a generous gift from Dr A Vecchi, Instituto di Ricerche Farmacologiche Mario Negri, Via Eritrea, Milan, Italy) (Vecchi et al., 1994), followed by incubation with biotinylated anti-rat IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) and the avidin-biotin complex ( Vectastain® ABC-AP Kit). Immunoglobulin complexes were visualised using Vector® Red alkaline phosphatase substrate solution.

Northern blot analysis

Total cellular RNA was extracted using RNAzol (Gibco, BRL) according to manufacturer’s instructions. RNA (10 μg) was denatured and electrophoresed in 1% agarose-formaldehyde gels as previously described (Ching et al., 1994). RNA was then transferred by capillary action onto nylon membrane (Hybond-N™, Amersham). The membranes were UV-crosslinked (120 mJoule, UV-Stratalinker, Stratagene, San Diego, CA, USA) and baked (80°C for 30 min). Each membrane was prehybridised (2 h, 42°C) in 7 ml hybridisation mix containing 50% formamide, 0.075 M sodium chloride, 0.05 M sodium dihydrogen phosphate, 5 mM EDTA, 0.001% polyvinyl pyrrolidone, 0.001% bovine serum albumin, 0.001% Ficoll, 0.01 mg ml⁻¹ herring sperm DNA, and 0.5% SDS (sodium dodecyl sulphate). The cDNA to the cytokine gene of interest was labelled with α³²P-dCTP (Amersham) using a random priming kit (RTS Radprime DNA labelling system, Gibco BRL). Excess radioactivity was removed by elution through a G-50 Sephadex column and labelled probe (10¹⁰ c.p.m. ml⁻¹ hybridisation) was then added to the membrane and hybridised for 36 h at 42°C. The blots were washed twice in 2×SSC (standard saline citrate) with 0.1% SDS for 10 min at 42°C, and finally in 0.2×SSC with 0.1% SDS for 10 min at 65°C. Blots were exposed to X-ray film for 1–3 days at −70°C. After hybridisation with one probe, membranes were stripped (two washes in 300 ml 0.1×SSC with 1% SDS for 15 min at 80°C) and re-hybridised with another probe. The signal intensity was quantitated by laser densitometric scanning. Loading of lanes was determined from the intensity of bands hybridised with the probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

RESULTS

Induction of endothelial cell apoptosis in murine tissues following DMXAA

Sections of Colon 38 tumours, removed at different times from C57Bl/6 mice administered DMXAA at its maximum tolerated dose (25 mg kg⁻¹), were double stained for apoptosis and for CD31 expression (Table 1). As early as 30 min after treatment, faint apoptotic staining above background staining of untreated controls (Figure 1A) was detected and found to be associated with the endothelial cells in the Colon 38 tumour (Figure 1B). The staining intensity, as well as the number of apoptotic vessels, increased progressively with time after treatment (Table 1). Up to 3 h after treatment, apoptosis was associated only with the tumour vascular endothelium, with no apoptosis of tumour cells (Figure 1B,C). Colon 38 sections taken 3 h after DMXAA administration contained intensely stained apoptotic vessels, and at this time, large areas of necrosis of the tumour were observed (Figure 1D). Following the maximal tolerated dose of DMXAA, 69% of the vessels in the tumour were apoptotic, while after a dose of 15 mg kg⁻¹, 27% were apoptotic. Significant apoptosis of vessels was not observed following DMXAA doses of 5 or 10 mg kg⁻¹ (Table 1). Colon 38 tumour sections removed from mice 3 h after treatment with an inactive analogue, 8-MeXAA (8-methylxanthenone-4-acetic acid) at its maximal tolerated dose (220 mg kg⁻¹) showed no apoptotic vessels or tumour necrosis (Figure 1E). No staining was observed with liver sections from either normal or tumour-bearing mice, before or 3 h after DMXAA (25 mg kg⁻¹) (Figure 1F). Strong staining for apoptosis was observed in the central vessel in the white pulp regions of spleens from all tumour-bearing mice 3 h after DMXAA (Figure 1G), and was not present without treatment. Apoptosis in the splenic vasculature was tumour-dependent, since apoptotic cells were not observed in spleens from normal mice following DMXAA administration (Figure 1H).
Endothelial cell apoptosis in human tumours following DMXAA

Tumour biopsies from one patient with breast adenocarcinoma prior to DMXAA infusion (3.1 mg m$^{-2}$) did not stain with the TUNEL assay (Figure 1I). However apoptotic staining in vessels was seen in the biopsy taken 3 h post-infusion (Figure 1I) and the number of apoptotic vessels increased three-fold in the biopsy taken after 24 h (Figure 1K; Table 1). In contrast with the murine 3 h samples, haemorrhagic necrosis was not observed. In another two patients administered DMXAA at 3.7 and 4.9 mg m$^{-2}$ no staining for apoptosis was seen in samples taken prior to drug infusion nor in breast carcinoma samples taken at 3 and 24 h or ovarian carcinoma samples taken at 4.5 h.

DMXAA induces HECPP endothelial cell apoptosis in culture

We used the murine endothelial HECPP cell line (Bizouarne et al., 1993) to determine whether DMXAA could induce apoptosis

Table 1 Quantitation of apoptotic vessels following DMXAA treatment

| Tumour/treatment | Dose (mg kg$^{-1}$) | Time (h) | n$^a$ | Apoptotic vessels (percentage)$^b$ | Apoptotic vessels per field$^c$ |
|------------------|--------------------|----------|-------|----------------------------------|---------------------------------|
| Colon 38         |                    |          |       |                                  |                                 |
| Untreated        | 25                  | 30 min   | 6     | 3.7 ± 1.5                        | 0.33 ± 0.21                     |
|                  | 25                  | 1 h      | 14    | 30 ± 4 (P < 0.001)               | 1.5 ± 0.2 (P < 0.005)           |
|                  | 25                  | 3 h      | 8     | 68 ± 8 (P < 0.001)               | 3.1 ± 0.8 (P < 0.005)           |
|                  | 15                  | 3 h      | 10    | 27 ± 3 (P < 0.001)               | 1.5 ± 0.2 (P < 0.001)           |
|                  | 10                  | 3 h      | 7     | 11 ± 4                            | 0.57 ± 0.20                     |
|                  | 5                   | 3 h      | 7     | 10 ± 5                            | 0.57 ± 0.20                     |
| 8-MeXAA          | 220                 | 3 h      | 2     | 1.8 ± 1.2                         | 0.33 ± 0.20                     |
| Human breast     |                    |          |       |                                  |                                 |
| Pre-treatment    | 3.1 m$^{-2}$        | 3 h      | 4     | N.D.$^d$                         | 0.0 ± 0.0                       |
| DMXAA            |                    | 3 h      | 4     | N.D.$^d$                         | 3.5 ± 0.9 (P < 0.001)           |
| DMXAA            | 3.1 m$^{-2}$        | 24 h     | 7     | N.D.$^d$                         | 9.1 ± 0.5 (P < 0.001)           |

$^a$Number of fields scored from different sections taken from 2 – 4 Colon 38 tumour-bearing mice or from the human tumour sections. $^b$Percentage of Colon 38 tumour vessels positive for the TUNEL assay. The total number of vessels was determined by staining with anti-CD-31 antibody. Mean ± s.e.m. Significance of difference from control is shown where P < 0.05 (Student’s $t$-test). $^c$Number of TUNEL-stained vessels per 1 mm$^2$ field, counted at 20 x magnification. Mean ± s.e.m. from Colon 38 tumour sections or from human breast carcinoma biopsies from the same patient before and after treatment with DMXAA. Significance of difference from control is shown where P < 0.05 (Student’s $t$-test). $^d$Not determined, since human tumour sections were not double-stained with anti-CD31 antibodies to quantitate the total number of vessels.

Figure 1 Induction of endothelial cell apoptosis by DMXAA. (A – E) Representative cryosections of Colon 38 tumours (100 x magnification) were immunostained for apoptosis using TUNEL (black) and endothelial cells with antibodies to CD-31 (red): (A) untreated, (B) 30 min after DMXAA (25 mg kg$^{-1}$), (C) 1 h after DMXAA, (D) 3 h after DMXAA, (E) 3 h after 8-MeXAA (220 mg kg$^{-1}$). (F – H) Representative cryosections (100 x magnification) immunostained for apoptosis using TUNEL (black): (F) liver tissue from Colon 38-bearing mice 3 h after DMXAA (25 mg kg$^{-1}$), (G) spleen tissue from Colon 38-bearing mice 3 h after DMXAA (25 mg kg$^{-1}$), and (H) spleen tissue from normal mice 3 h after DMXAA (25 mg kg$^{-1}$). (I – L) Representative cryosections (100 x magnification) from a human breast carcinoma immunostained for apoptosis using TUNEL (black): (I) before treatment, (J) 3 h after DMXAA infusion (3.1 mg m$^{-2}$), (K) 24 h after DMXAA. (L) Section at higher magnification (500 x) of two vessels 3 h after DMXAA infusion.
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in vitro in the absence of TNF induction. As seen from Northern blot analyses, DMXAA does not induce mRNA for TNF or interferons in the HECPP cells (Figure 2A). Of the cytokine genes that are up-regulated in mice following DMXAA treatment (Figure 2B), only mRNA for IP-10 was up-regulated following 2 h incubation with DMXAA at 400 μg ml⁻¹ in the HECPP cells (Figure 2A). HECPP cells treated with DMXAA were processed using TUNEL assays and the percentages of apoptotic cells were determined by counting at least 500 cells. Following exposure to DMXAA at a concentration (400 μg ml⁻¹) that is achievable in vivo after administration of an effective antitumour dose (McKeage et al, 1991), apoptotic cells were seen after 6 h incubation, and the numbers increased with prolonged exposure (Figure 3A). Apoptotic cell numbers at 24 h increased linearly with increasing dose of DMXAA above 100 μg ml⁻¹. The DMXAA concentration that induced 50% apoptosis after incubation for 24 h was 500 μg ml⁻¹ (Figure 3B).

DISCUSSION

These results are the first to demonstrate the selective induction of endothelial cell apoptosis in both a murine tumour (Figure 1B–D) and a human tumour (Figure 1F–L) following treatment with DMXAA. Loss of tumour vascular endothelial cells by apoptosis would be expected to increase the permeability of the vascular endothelium, providing a potential mechanism for reduction in tumour blood flow (Baguley, 2001) as demonstrated in both murine models (Zwi et al, 1994a,b; Lash et al, 1998) and in clinical studies (Rustin et al, 1998). The results also provide a possible mechanism for the DMXAA-induced extravasation of erythrocytes in murine tumours (Zwi et al, 1994b). Although a number of studies have implicated TNF in the antitumour response of DMXAA (Browne et al, 1998; Cao et al, 1999), three observations in this study support a TNF-independent action of DMXAA on the vascular endothelium. Firstly, apoptosis of endothelial cells was observed in Colon 38 tumour sections with-
tioned growth medium from a cultured human breast adenocarcinoma cell line. The tumour conditioned medium had a greater effect on endothelial cells than did hypoxia, reduced oxygen tension, TNF, FAA or DMXAA (Watts et al., 1996), providing another clear example of the priming effect of tumour secreted factors.

The ‘priming’ effect may provide an explanation of the remarkably rapid onset (30 min) of apoptosis of vascular endothelial cells in the Colon 38 tumour. It is not known whether vascular endothelial apoptosis could occur as rapidly in human tumours, since the earliest time point following DMXAA that we were able to examine was 3 h. However, apoptosis occurred more rapidly following DMXAA administration in vivo than in HECPP cells in culture.

Clinical trials of DMXAA have indicated a low response rate to DMXAA (Jameson et al., 2000). However, good evidence has been provided for induced vascular effects, such as reduced tumour blood flow (Rustin et al., 1998), serotonin release (Jameson et al., 2000) and the endothelial apoptosis reported in this study. The clinical trial results differ from those of murine studies in that although TNF synthesis was detected in one human tumour sample (Jameson et al., 2000) the level was low and TNF was not detected in the sample in which endothelial cell apoptosis was observed.

Widespread haemorrhagic necrosis is a prominent effect of DMXAA treatment in murine tumours (Baguley et al., 1989; Zwi et al., 1994b; Cao et al., 1999) but has not so far been observed in clinical trials. In the Colon 38 tumour, in situ TNF synthesis (Joseph et al., 1999) is probably responsible for sustaining the vascular effect of DMXAA and inducing haemorrhagic necrosis. Combination of DMXAA with agents that improve intratumoural TNF synthesis might constitute a rational approach in further clinical trials of DMXAA. In cultured human peripheral blood leucocytes, a second signal, such as that provided by trace amounts of endotoxin or interleukin-1, is required in order for DMXAA to induce TNF production (Philpott et al., 2001). Clinical studies employing DMXAA in combination with other agents that provide a second signal within tumour tissue may provide a pathway towards improved anticancer therapy.

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