Melanoma Research 2000, **10**, pp. 435–443

Theophylline administration markedly reduces hepatic and pulmonary implantation of B16-F10 melanoma cells in mice

A. Lentini, F. Vidal-Vanaclocha, F. Facchiano, M. Caraglia, A. Abbruzzese and S. Beninati*

Department of Biology, University 'Tor Vergata', Via della Ricerca Scientifica, 00133 Rome, Italy. Tel: (+39) 6 72594350; Fax: (+39) 6 2023500; Email: beninati@bio.uniroma2.it (A. Lentini, S. Beninati). Department of Cellular Biology and Morphological Sciences, University of the Basque Country, Leioa, Vizcaya, Spain (F. Vidal-Vanaclocha). Laboratory of Vascular Pathology, Istituto Dermopatico dell'Immacolata, Rome, Italy (F. Facchiano). Department of Biochemistry and Biophysics, University of Naples, Italy (M. Caraglia, A. Abbruzzese).

Theophylline-treated B16-F10 melanoma cells show a lower experimental metastatic potential *in vivo*. To identify the possible mechanism(s) involved and on the basis of previous reports, we tested the induction of apoptosis in B16-F10 cells. Fluorescence activated cell sorter (FACS) analysis and p53 overexpression in theophylline-treated B16-F10 melanoma cells appeared to suggest enhanced cell death by apoptosis. The *in vivo* effects of orally administered theophylline in mice were investigated using different treatment schedules in mice that had undergone hepatic or pulmonary colonization with tumour cells. Mice received theophylline in their drinking water according to different protocols: (i) from 3 days before tumour cell inoculation until animal sacrifice ('early treatment'); (ii) from 3 days before until 3 days after tumour cell inoculation ('short treatment'); or (iii) from 3 days after tumour cell inoculation until animal sacrifice ('late treatment'). In the 'early treatment' group, the number of melanoma foci was reduced by 92.3% in the liver and 81.4% in the lung compared with control animals ($P < 0.001$). In the 'short treatment' group, there was an 80.2% and 72.2% reduction in liver and lung metastases, respectively ($P < 0.001$). In the 'late treatment' group, the inhibition of metastasis was 59.7% for liver and 45.3% for lung ($P < 0.005$). Survival studies showed that 50% of the 'early' theophylline-treated animals died 33.2 ± 2.0 days after intrasplenic injection (control group: 23.1 ± 1.8 days; $P < 0.001$) and 33.9 ± 2.5 days after tail vein injection (control group: 24.1 ± 1.4 days; $P < 0.001$). Taken together, these observations provide useful information for the potential clinical application of theophylline as a chemotherapeutic agent against malignant melanoma. © 2000 Lippincott Williams & Wilkins

Key words: melanoma cells, metastasis, methylxanthines

*To whom correspondence should be addressed

Introduction

The migration of malignant tumour cells from a primary site to near and distant secondary sites is probably the most important event in the pathogenesis of cancer. Tumour metastatization involves a sequence of events that culminates in the dissemination and establishment of metastatic foci.[1,2] During the haematogenous phase of the metastatic process, tumour cells undergo a variety of cell-cell interactions beneficial to tumour cell survival, such as interaction with platelets and attachment to vascular endothelium[3] and basement membrane proteins.[4,5] Moreover, target tissue colonization involves local proteolysis, migration and cellular growth.[6]

The key role of the environment of a neoplasm in affecting its metastatic or malignant behaviour is well established.[7] In particular, the malignancy of diverse tumours, including melanoma, can be markedly altered by conditions that induce cell differentiation.[8,9] The differentiation of melanoma cells from unpigmented to black, melanin-synthesizing cells is understood in some biological and biochemical detail,[10] and can be induced in cultured melanoma cells by various agents. Most of these agents are known to enhance the intracellular activity of transglutaminase, including tetanus toxin,[11] theophylline[12] and retinoic acid.[13] Methylxanthines, and in particular theophylline, can induce differentiation, paralleled with a reduction in proliferation, in B16 melanoma cells.[14,15] Some of the effects of these natural compounds are of interest because of

the widespread use of methylxanthine-containing beverages (coffee, tea, etc.), whereas others have been used to therapeutic advantage.[16,17] It is well known that theophylline acts as cAMP-phosphodiesterase inhibitor, involving alterations in the cAMP system of tumour cells.[18] Moreover, many of the biological effects of adenosine can be reverted by theophylline, suggesting a role as an adenosine antagonist.[19] More recently a role for theophylline has been suggested in the induction of apoptosis in blood cells,[20,21] whereas possible effects on melanoma cell lines are as yet lacking. We have recently shown that theophylline remarkably affected the *in vitro* migration and invasion of B16-F10 melanoma cells through reconstituted basement membranes, and reduced the size and frequency of pulmonary colonies after intravenous injection of tumour cells into syngeneic mice. Moreover, the pretreatment of tumour cells with theophylline reduced their adhesion to specific basement membrane proteins, i.e. laminin and collagen type IV.[14,22]

The aim of the present work was to extend our knowledge about the mechanism(s) involved in theophylline-mediated antineoplastic activity, investigating the possible induction of apoptosis in B16-F10 melanoma cells treated with theophylline using flow cytometric evaluation of DNA fragmentation and analysis of p53 overexpression. We then studied the influence of theophylline treatment on tumour implantation after melanoma cell inoculation in mice. The invasive capacity of B16-F10 cells in mice treated with oral theophylline was evaluated in two different experimental models: lung colonization, via tail vein injection, and liver colonization, via intrasplenic inoculation.

Materials and methods

Materials

Theophylline was purchased from Sigma Chemicals Co. (St Louis, Missouri, USA). Dulbecco's modified essential medium (DMEM), fetal calf serum (FCS) and Versene (EDTA) were from Gibco Laboratories (Grand Island, New York, USA). All other chemicals were from Merck (Darmstadt, Germany).

Cell cultures

B16-F10 murine melanoma cells (obtained from I.J. Fidler, University of Texas, MD Anderson Cancer Centre, Houston, Texas, USA) were propagated under standard culture conditions.[23] Cultures were shown to be free from *Mycoplasma* species using the Hoechst staining procedure. The metastatic potential of the cell line has been assessed in an *in vitro* assay, as described elsewhere.[24] The metastatic activity, both spontaneous and experimental, of this line was evaluated and was found to be stable over the time of the experimental procedure performed.

Flow cytometric cell cycle analysis and evaluation of apoptosis

Melanoma cells were plated onto six-well plates (1×10^5 cells), treated with theophylline (0.1 mM, 0.5 mM or 1 mM) for 48 h, then detached, washed once with phosphate buffered saline (PBS) and fixed in 70% cold ethanol. The fixed cells were then washed with PBS containing 1% FCS, incubated with 50 µg/ml ribonuclease A (RNase A, Sigma Chemicals Co.) for 30 min at 37°C and stained with propidium iodide (PI) (50 µg/ml, Sigma Chemicals Co.) according to standard protocols and gated out electronically. The relative DNA content and percentage of cells in various cell cycle compartments were determined using a FACScan (Becton Dickinson) and CellQuest software. The percentage of apoptotic cells was calculated by selecting cells exhibiting a mean channel fluorescence (MCF) below the G_1phase of the cell cycle.[25] DNA fragments smaller in size than 10% of those corresponding to G_1 cells were not included in the analysis, according to published protocols.[26]

Western blot and densitometry

Cells were cultured and treated with theophylline as described before. They were then detached, 100 µg of total proteins added with Laemmli solution, boiled, and electrophoresed in 8–15% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and electroblotted to a nitrocellulose membrane (Biorad). The membrane was blocked with 3% gelatine solution, then incubated with 0.2 µg/ml antibody to p53 (monoclonal Pab-240, Santa Cruz Biotechnology Inc.) for 90 min at room temperature. Finally, immunoreactive p53 was detected using the ECL detection system (Amersham Life Technologies), according to the manufacturer's instructions. For quantification, densitometric analysis was performed using a Gel-Pro analyser (Media Cybernetics, USA),

and the integrated optical density (IOD) values were expressed as a percentage of the control (100%).

Animals

Male 6 week old C57BL/6N mice were obtained from IFFA Credo (L'Abresle, France) and housed under pathogen-free conditions. All experimental protocols were carried out following the Guidelines for the Welfare of Animals in Experimental Neoplasia (*Br J Cancer* 1998; 77(1): 1–10) and the ECC Council Directive 86/609, OJL 358, 1st December 1987.

Theophylline treatment protocols

Mice were treated orally with 20 mM theophylline in tap water, according to different protocols: (i) from 3 days before tumour cell inoculation until animal sacrifice ('early treatment'); (ii) from 3 days before until 3 days after tumour cell inoculation ('short treatment'); or (iii) from 3 days after tumour cell inoculation until animal sacrifice ('late treatment'). Plasma levels of theophylline were measured using high performance liquid chromatography (HPLC), as described elsewhere.[27] Theophylline concentrations were extrapolated from a standard curve. Daily plasma extractions were carried out in triplicate.

Quantitative evaluation of liver and lung metastasis

For lung experimental metastasis, viable B16-F10 cells were detached with EDTA, counted, resuspended in DMEM at a concentration of 10^6 cells/ml and 200 μl injected into the tail vein of anaesthetized mice. Hepatic metastases were produced by the intrasplenic injection of 2×10^5 tumour cells suspended in DMEM (10^6 cells/ml). As a control, untreated or theophylline-treated mice were injected with vehicle only. A second control group received no treatment, but was inoculated with tumour cells. Animals were sacrificed by cervical dislocation 10 days after intrasplenic inoculation and 15 days after intravenous inoculation. Livers and lungs were rapidly excised, blotted, weighed and processed for histology. Evaluation of the metastatic activity was obtained by a computerized morphometric method.[28] About 15 tissue cryosections were obtained per organ: three 4 μm sections were made every 100 μm from each of five groups approximately 500 μm apart. Sections were stained with haematoxylin and eosin. Densitometric analysis of digitalized microscopic images was used to discriminate metastatic tissue from normal tissue and to determine the average number of metastatic foci per mm^3 of tissue, as described previously.[28] There were 20 animals in each experimental group.

For the survival studies, about 10 untreated or theophylline-treated mice were injected intrasplenically or via the tail vein with B16-F10 cells. Each experiment was repeated twice. Moribund animals were killed without waiting for their predictable death, according to UKCCCR guidelines.

Statistical analysis

Data were analysed using the Student's unpaired t-test. Kaplan-Meier survival curves were used for the mortality studies. Differences were considered to be significant at P values < 0.05.

Results

Fluorescence activated cell sorter (FACS) analysis of B16-F10 cells treated with theophylline

Cells treated with theophylline for 48 h were subjected to flow cytometric evaluation of DNA fragmentation. The percentage of cells with a DNA content below the G_1 peak (sub-G_1) was calculated to give the M1/M2 ratio. Figure 1 shows untreated B16-F10 cells (M1/M2 = 0.042, Figure 1A), cells treated with 0.1 mM theophylline (M1/M2 = 0.195, Figure 1B), cells treated with 0.5 mM theophylline (M1/M2 = 0.356, Figure 1C) and cells treated with 1 mM theophylline (M1/M2 = 0.797, Figure 1D). These results show an enhancement of the M1/M2 ratio with an increase in theophylline concentration.

Expression of p53

Cell extracts from 1.5×10^6 B16-F10 melanoma cells, preincubated with theophylline as described, were processed for Western blot analysis for p53 detection. As shown in Figure 2, the amount of p53 appeared to increase in cells treated with 1 mM theophylline (lane D, IOD = 320%) compared with the control (lane A, IOD = 100%), whereas smaller changes were seen after treatment with 0.1 mM theophylline (lane B, IOD = 123%) or 0.5 mM theophylline (lane C, IOD = 143%).

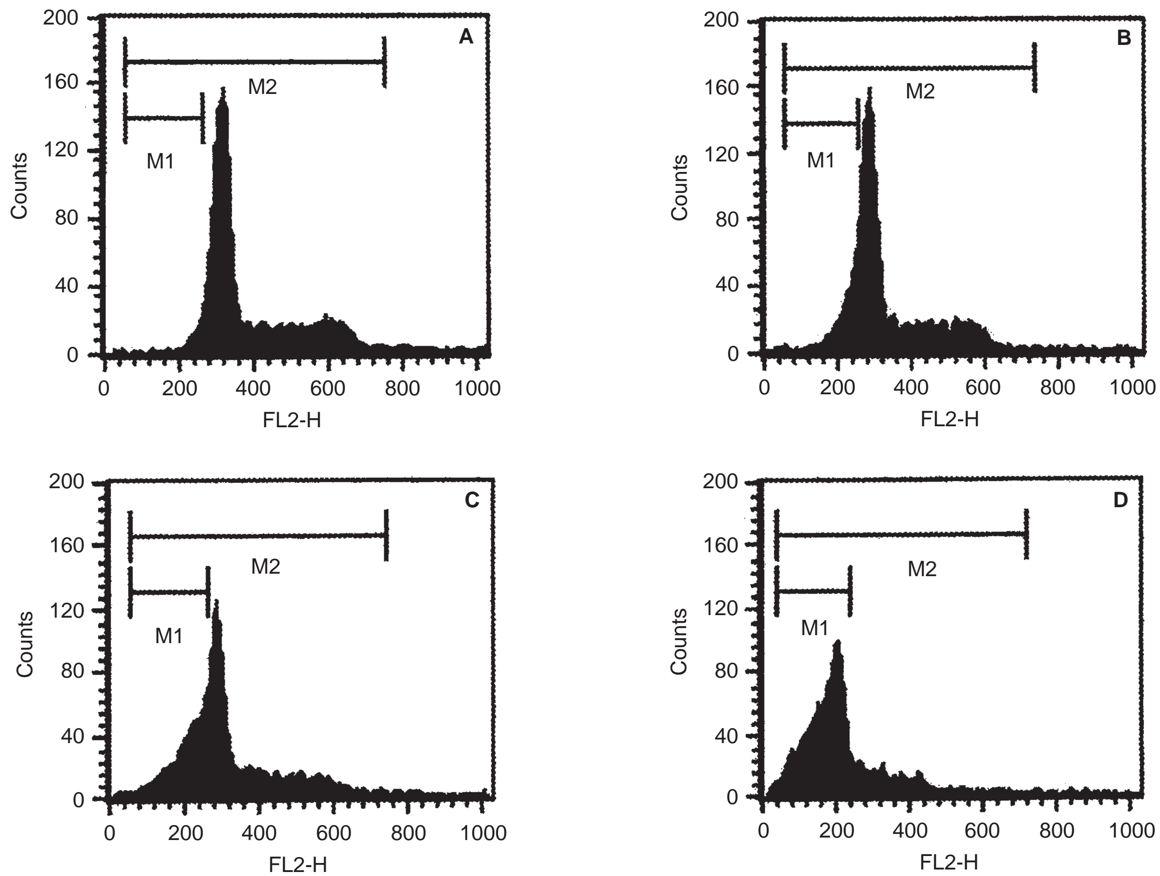

Figure 1. FACS analysis of B16-F10 melanoma cells untreated (**A**) or treated with 0.1 mM (**B**), 0.5 mM (**C**) or 1 mM (**D**) theophylline for 48 h, as described in Materials and methods. The experiment was repeated twice in duplicate with similar results. The data shown come from a representative experiment.

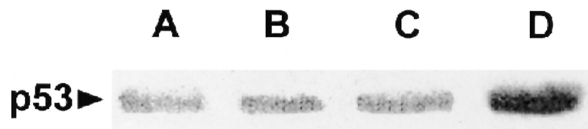

Figure 2. Western blot analysis of p53 in untreated and theophylline-treated B16-F10 melanoma cells, as described in Materials and methods. The experiment was repeated twice with similar results. Lane A, control, IOD = 100%; lane B, 0.1 mM theophylline, IOD = 123%; lane C, 0.5 mM theophylline, IOD = 143%; lane D, 1 mM theophylline, IOD = 320%.

Detection of plasma levels of theophylline

Figure 3 shows that the administration of 20 mM theophylline in tap water to mice leads to a steady concentration of this compound in the blood (about 100 μM) after 3 days. These plasma levels of theophylline are lower than those considered to be toxic (300 μM) by LD_{50} determination (data not shown).

Effect of theophylline treatment on B16-F10 metastasis

Due to the highly metastatic nature of B16-F10 cells, implantation into the target organ is rapid following intrasplenic or intravenous injection. After intrasplenic injection a tumour is produced in the spleen, and all mice develop hepatic metastases. By the fourth day a visible melanotic tumour (10 mm^3) is present in the spleen, which rapidly grows to a diameter of about 2–3 cm. Liver metastases are detectable microscopically from the seventh day, and half of the liver volume is occupied by tumour colonies by the tenth day from cell inoculation. Following intravenous injection of B16-F10 cells via the tail vein, no primary tumours were produced at the site of inoculation. Pulmonary metastases were visible from the eighth day after the injection as single black spots.

The influence of theophylline treatment of mice on the experimental metastasis of B16-F10 melano-

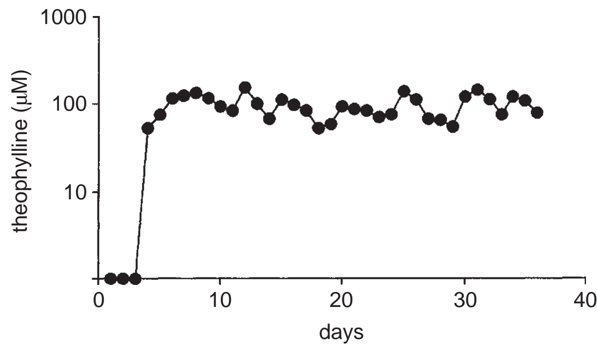

Figure 3. HPLC determination of theophylline concentration in the blood of C57BL/6N mice orally fed with 20 mM theophylline in tap water. Each point represents the mean of three determinations obtained from three different mice. For all points, the standard deviation did not exceed 10% of the mean value.

ma was investigated according to different administration schedules. Three groups of mice were treated orally with theophylline: (i) the 'early treatment' group, (ii) the 'short treatment' group', and (iii) the 'late treatment' group (see above). Untreated animals were injected with melanoma cells on the same day as the treated animals.

As shown in Table 1, tumour cells injected into mice caused an increase in liver (1.7-fold) and lung (2.8-fold) weight compared with normal organs ($P < 0.001$) due to metastases formation, as confirmed by histological analysis (data not shown). In theophylline-treated mice ('early treatment' group), a 1.2-fold increase in liver weight and a 1.7-fold increase in lung weight ($P < 0.01$) occurred, even when the treatment was stopped 3 days after tumour cell injection ('short treatment' group). When theophylline administration began 3 days after cell injection ('late treatment' group) the increases in weight were 1.5-fold for liver and 2.6-fold for lung ($P < 0.005$). The treatment of mice with theophylline without tumour cell injection did not increase the weight of the target organs.

Table 2 shows the effect of the different schedules of theophylline administration on metastasis frequency. When mice were treated with theophylline for 3 days before tumour inoculation ('early treatment' group), both the hepatic and pulmonary metastatic density decreased markedly compared with control (92.3% and 81.4% reduction, respectively; $P < 0.001$). When the oral treatment was interrupted 3 days after the injection of B16-F10 cells ('short treatment' group), we observed a 80.2% reduction in hepatic tumour density and a 72.2% reduction in pulmonary metastasis compared with untreated animals ($P < 0.001$). When the oral treatment was started on the third day after the inoculation of cells ('late treatment' group), a 59.7%

Table 1. Effect of theophylline treatment on the liver and lung weights of mice injected with B16-F10 melanoma cells

Theophylline treatment	Inoculation of B16-F10 cells	Liver weight (g)	Lung weight (g)	% increase in liver weight	% increase in lung weight
None	No	1.24 ± 0.11	0.18 ± 0.01	—	—
'Early'	No	1.20 ± 0.09	0.20 ± 0.01	—	—
None	Yes	2.07 ± 0.17^{a}	0.50 ± 0.03^{a}	66.9^{a}	177.7^{a}
'Early'	Yes	1.47 ± 0.13^{b}	0.30 ± 0.02^{b}	18.5^{b}	66.7^{b}
'Short'	Yes	1.49 ± 0.15^{b}	0.35 ± 0.04^{b}	20.2^{b}	94.4^{b}
'Late'	Yes	$1.79 \pm 0.15^{c,d}$	$0.46 \pm 0.03^{c,d}$	$44.3^{c,d}$	$155.5^{c,d}$

[a] $P < 0.001$ between mice not inoculated with cells and inoculated mice (control).
[b] $P < 0.01$ between 'early' or 'short' treatments and control.
[c] $P < 0.005$ between 'late' treatment and control.
[d] $P < 0.001$ between 'late' and 'early' treatments.

Table 2. Effect of theophylline treatment on liver and lung metastasis parameters

Theophylline treatment	Number of liver metastases/mm^3	Number of lung metastases/mm^3	% liver metastasis reduction	% lung metastasis reduction
None	30.02 ± 7.12	13.72 ± 0.91	—	—
'Early'	2.30 ± 0.81^{a}	2.55 ± 0.20^{a}	92.3^{a}	81.4^{a}
'Short'	5.95 ± 2.05^{a}	3.82 ± 0.28^{a}	80.2^{a}	72.2^{a}
'Late'	$12.10 \pm 3.32^{b,c}$	$7.50 \pm 0.44^{b,c}$	$59.7^{b,c}$	$45.3^{b,c}$

[a] $P < 0.001$ between 'early' or 'short' treatments and control.
[b] $P < 0.005$ between 'late' treatment and control.
[c] $P < 0.001$ between 'late' and 'early' treatments.

reduction in the number of liver foci and a 45.3% reduction in lung colonization was seen ($P < 0.005$).

Effect of theophylline on survival

The effect of oral treatment with theophylline on the survival of mice injected intrasplenically or intravenously with B16-F10 melanoma cells was investigated. Animals were orally fed with 20 mM theophylline in tap water for 3 days prior to the inoculation of cells. As shown in Figure 4, 'early treatment' with theophylline caused a long-term (8–10 days) delay in mortality; 50% of the theophylline-treated animals were dead by 33.2 ± 2.0 days after intrasplenic injection of tumour cells (control group: 23.1 ± 1.8 days; $P < 0.001$) (Figure 4A) and 33.9 ± 2.5 days after tail vein injection (control group: 24.1 ± 1.4; $P < 0.001$) (Figure 4B). The 'short treatment' and 'late treatment' with theophylline had a smaller effect on the life span of the animals (data not shown).

Discussion

Theophylline has previously been demonstrated to be the most effective methylxanthine to inhibit the experimental metastasis of B16-F10 melanoma.[14] Using flow cytometric analysis of DNA fragmentation and Western blot analysis of p53 expression, we studied the eventual induction of apoptosis in theophylline-treated cells in the light of previous work concerning other cell lines.[20,21,29] Theophylline treatment of B16-F10 cells appears to induce an increase in the number of cells arrested in the G_1 phase (Figure 1) and an enhancement of the expression of p53 (Figure 2). These findings strongly support a possible involvement of apoptosis in the antineoplastic activity of theophylline, as previously observed in other cells.[30]

On the basis of these *in vitro* results, the *in vivo* effect of theophylline on experimental lung and liver metastasis of B16-F10 murine melanoma cells was further investigated. Since many of the morphological and functional effects induced *in vitro* on tumour cells by theophylline are known to be readily reversible,[31] the inoculation of pretreated melanoma cells in syngeneic mice, without the continued administration of the drug, is not likely to reveal the full potential of theophylline in tumour cells *in vivo*. A full evaluation of the *in vivo* utility of this agent will require that the bioactive concentration is achieved in the target tissues and is maintained long enough to produce pharmacodynamic effects. Such *in vivo* evaluation necessarily needs knowledge concerning the formulation, administration, and dose-limiting host toxicity as a function of formulation, route of administration and dose scheduling. Although oral administration of theophylline appeared to be an effective route for inhibiting melanoma metastasis, it was obviously difficult to monitor the amount of drug consumed by mice. To overcome this difficulty, the plasma levels of theophylline were determined daily using

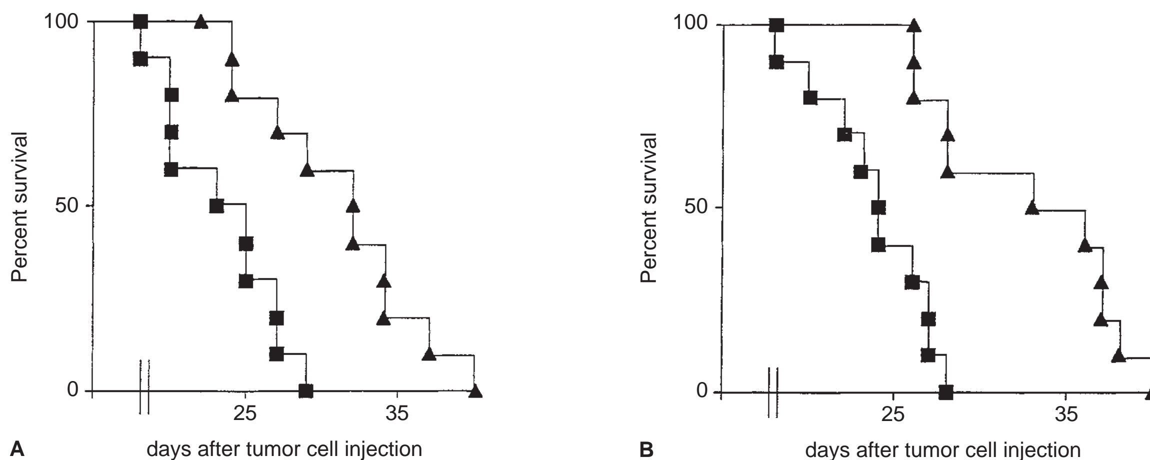

Figure 4. Effect of theophylline administration on the survival of B16-F10 hepatic or pulmonary metastasis-bearing mice. Kaplan-Meier survival curves for untreated mice (■, $n = 10$) and mice fed with 20 mM theophylline in tap water (▲, $n = 10$) for 3 days before inoculation of tumour cells ('early treatment'). **A** The average 50% survival time was 33.2 ± 2.0 days after intrasplenic injection of tumour cells (producing hepatic tumour) into theophylline-treated mice (control group: 23.1 ± 1.8 days; $P < 0.001$, unpaired t-test). **B** The average 50% survival time was 33.9 ± 2.5 days after tail vein injection of tumour cells (producing pulmonary tumour) into theophylline-treated mice (control group: 24.1 ± 1.4 days; $P < 0.001$, unpaired t-test).

HPLC (Figure 3). Theophylline reached a peak plasma concentration at about the third day of treatment, and this value was found to then be stable with time. It is noteworthy that the serum levels of theophylline were two to three times lower than the toxic concentration ($LD_{50} = 300\ \mu M$), suggesting that the observations reported here are not due to cytotoxic effects. Furthermore, theophylline treatment alone did not exert any effect on the liver and lung weights (Table 1). We have shown that theophylline treatment of B16-F10 melanoma-bearing mice reduced tumour implantation in two different organs (i.e. lung and liver) to various extents, depending on the time of the administration of the drug. When theophylline treatment began very early with respect to the injection of cells ('early treatment'), the average frequency of lung and liver colonies was greatly reduced compared with untreated animals. However, stopping the treatment after 3 days ('short treatment') or starting it 3 days after the injection of tumour cells ('late treatment') produced a smaller effect on the colony-forming capacity of B16-F10 cells. These results suggest that invasion of the target organ by melanoma cells is a very fast and lasting process. The presence of theophylline in the blood of animals during the inoculation of melanoma cells ('early treatment' and 'short treatment' groups) appears critical for greater inhibition of tumour cell invasion. On the other hand, since secondary metastases probably contribute to the total frequency of tumour foci, the late action of theophylline after the inoculation of melanoma cells may account for the observed effects in the 'late treatment' group. Independent of the timing of administration, theophylline had a greater inhibitory effect on B16-F10 cell colonization of the liver when compared with that observed in the lung. This finding is particularly interesting considering that the liver of untreated mice had the highest frequency of metastases (Table 2). We suggest that the enhanced hepatic susceptibility to theophylline treatment as well as the increased invasiveness of B16-F10 cells may be related to the well-known absence of the hepatic endothelial basement membrane.[32] Analysis of the survival curves clearly indicates that 'early treatment' significantly affects the life span of hepatic or pulmonary tumour-bearing mice (Figure 4). Although this finding may simply derive from the 3 days required for the drug to reach the active plasma concentration, studies are in progress to investigate the higher efficacy of theophylline in mice treated before tumour cell injection.

With regard to the mechanisms for the effect of theophylline on tumour cell proliferation and invasion, some possibilities will be mentioned. Inhibitory effects of cAMP or its analogues on tumour cells *in vitro*, as well as a reduction in their malignant phenotypic characteristics, have been reported.[33–35] It is therefore tempting to ascribe the inhibitory effects of theophylline on B16-F10 melanoma cells to alterations in the endogenous cAMP system of the cells and to relate them to the inhibitory effects of exogenous cAMP noted by others. However, direct data documenting such a relationship are as yet lacking.

We recently reported that theophylline was able to reduce the *in vitro* invasive and proliferative capability of B16-F10 cells, probably via the induction of cell differentiation.[22] Differentiation therapy for human neoplastic diseases remains an elusive, although quite likely a realistic goal. For instance, most patients with acute promyelocytic leukaemia achieve complete remissions when they are treated with all-*trans*-retinoic acid,[36,37] and more recently a therapeutic efficacy for theophylline in chronic lymphocytic leukaemia was reported.[38] We observed that theophylline treatment led to enhanced p53 expression in B16-F10 cells, indicating the induction of apoptosis. This finding was also confirmed by FACS analysis. We are collecting immunohistochemical evidence of the increase of p53 expression in metastatic foci found in B16-F10 and SK-MEL target organs from theophylline-treated mice (data not shown), which supports the *in vivo* induction of apoptosis. Since retinoic acid, as well as theophylline, are effective activators of transglutaminases, a class of enzymes involved in cell differentiation[39–41] and apoptosis,[42,43] it is reasonable to hypothesize that these enzymes may be involved in the molecular events leading to tumour cell differentiation and/or cell death.

Acknowledgements

We would like to thank Professor Francesco Autuori (Department of Biology, University 'Tor Vergata', Rome, Italy) for valuable discussions throughout these investigations, and Miss Giulia Patrizi for her skilful technical assistance. This work was supported by grants from the Consiglio Nazionale delle Ricerche (target project 'Invecchiamento' no. 9200339) and from the University of Rome 'Tor Vergata' (project 'Giovani Ricercatori' no. 20948).

References

1. Fidler IJ, Gersten DM, Hart IR. The biology of cancer invasion and metastases. *Adv Cancer Res* 1978; **28**: 149–250.
2. Weiss L. Overview of the metastatic cascade. In: Honn KW, Sloane BF, eds. *Hemostatic Mechanism and Metastasis*. Boston: Martinus Nijhoff, 1984: 15–38.
3. Barberà-Guillem E, Vidal-Vanaclocha F. Selective involvement of a specific sinusoidal domain in hepatic metastasis. In: Orr FW, Buchanan MR, Weiss L, eds. *Microcirculation in Cancer Metastasis*. Boca Raton, Florida: CRC Press, 1991: 183–203.
4. Alford D, Pitha-Rowe P, Taylor-Papadimitriou J. Adhesion molecules in breast cancer: role of alpha2-beta1 integrin. *Biochem Soc Symp* 1998; **63**: 245–259.
5. Fukushima Y, Ohnishi T, Arita N, Hayakawa T, Sekiguchi K. Integrin alpha3beta1-mediated interaction with laminin-5 stimulates adhesion, migration and invasion of malignant glioma cells. *Int J Cancer* 1998; **76**(1): 63–72.
6. Reuning U, Magdolen V, Wilhelm O, Fischer K, Lutz V, Graeff K, Schmitt M. Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis. *Int J Oncol* 1998; **13**(5): 893–906.
7. Paget S. The distribution of secondary growth in cancer of the breast. *Lancet* 1989; **i**: 571–573.
8. Tsukamoto K, Gersten DM, Law LW, Hearing VJ. Malignant melanoma: relationship to parameters of differentiation. *Melanoma Res* 1991; **1**: 223–230.
9. Li ZR, Hromchak R, Mudipalli A, Bloch A. Tumor suppressor proteins as regulators of cell differentiation. *Cancer Res* 1998; **58**(19): 4282–4287.
10. Bennett DC. Genetics, development and malignancy of melanocytes. *Int Rev Cytol* 1993; **146**: 191–260.
11. Facchiano F, Luini A. Tetanus toxin potently stimulates tissue transglutaminase. A possible mechanism of neurotoxicity. *J Biol Chem* 1992; **267**(19): 13267–13271.
12. Beninati S, Abbruzzese A, Cardinali M. Differences in the post-translational modification of proteins by polyamines between weakly and highly metastatic B16 melanoma cells. *Int J Cancer* 1993; **53**: 792–797.
13. Joseph B, Lefebvre O, Mereau-Richard C, Danze PM, Belin-Plancot MT, Formstecher P. Evidence for the involvement of both retinoic acid receptor- and retinoic X receptor-dependent signaling pathways in the induction of tissue transglutaminase and apoptosis in the human myeloma cell line RPMI 8226. *Blood* 1998; **91**(7): 2423–2432.
14. Lentini A, Kleinman HK, Mattioli P, Autuori-Pezzoli V, Nicolini L, Pietrini A, Abbruzzese A, Cardinali M, Beninati S. Inhibition of melanoma pulmonary metastasis by methylxanthines due to decreased invasion and proliferation. *Melanoma Res* 1998; **8**(2): 131–137.
15. Supino R, Mariani M, Colombo A, Prosperi E, Croce AC, Bottiroli G. Comparative studies on the effects of doxorubicin and differentiation inducing agents on B16 melanoma cells. *Eur J Cancer* 1992; **28A**(4–5): 778–783.
16. Persson C. Some pharmacological aspects on xanthines in asthma. *Eur J Respir Dis Suppl* 1980; **109**(61): 7–16.
17. Rabe KF, Dent G. Theophylline and airway inflammation. *Clin Exp Allergy* 1998; **28**(suppl 3): 35-41.
18. Bergstrand H. Phosphodiesterase inhibition and theophylline. *Eur J Respir Dis Suppl* 1980; **109**(61): 37–43.
19. Fredholm BB. Theophylline actions on adenosine receptors. *Eur J Respir Dis Suppl* 1980; **109**(61): 29–36.
20. Yasui K, Nakazawa T, Agematsu K, Komiyama A. Theophylline accelerates human granulocyte apoptosis not via phosphodiesterase inhibition. *J Clin Invest* 1997; **100**(7): 1677–1684.
21. Mentz F, Merle-Beral H, Dalloul AH. Theophylline-induced B-CLL apoptosis is partly dependent on cyclic AMP production but independent of CD38 expression and endogenous IL-10 production. *Leukemia* 1999; **13**(1): 78–84.
22. Lentini A, Mattioli P, Nicolini L, Pietrini A, Abbruzzese A, Beninati S. Anti-invasive effects of theophylline on experimental B16-F10 melanoma lung metastasis. *Cancer J* 1997; **10**(5): 274–278.
23. Fidler IJ. Selection of successive tumor lines for metastases. *Nature New Biol* 1973; **245**: 148–149.
24. Albini A, Iwamoto Y, Kleinman HK. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987; **47**: 3239–3245.
25. Telford WG, King LE, Fraker PJ. Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. *Cytometry* 1992; **13**: 137–143.
26. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 1997; **27**: 1–20.
27. Edholm LE. Specific methods for theophylline assay in biological samples. *Eur J Respir Dis Suppl* 1980; **61**: 45–53.
28. Vidal-Vanaclocha F, Alonso-Varona A, Ayala R, Boyano MD, Barberà-Guillem E. Coincident implantation, growth and interaction sites within the liver of cancer and reactive hematopoietic cells. *Int J Cancer* 1990; **46**: 267–271.
29. Kim DH, Lerner A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. *Blood* 1998; **92**(7): 2484–2494.
30. Mentz F, Mossalayi MD, Ouaaz F, Baudet S, Issaly F, Ktorza S, Semichon M, Binet JL, Merle-Beral H. Theophylline synergizes with chlorambucil in inducing apoptosis of B-chronic lymphocytic leukemia cells. *Blood* 1996; **88**(6): 2172–2182.
31. Beninati S, Mantile G, Cardinali M. Biochemical regulation of transglutaminase activity in murine melanoma cells with different metastatic properties: effect of retinoic acid and methylxanthines. In: Livrea MA, Parker L, eds. *Retinoids: Progress in Research and Clinical Applications*. New York: Marcel Dekker, 1993: 341–360.
32. Nonaka Y, Iwagaki H, Kimura T, Fuchimoto S, Orita K. Effect of reactive oxygen intermediates on the in vitro invasive capacity of tumor cells and liver metastasis in mice. *Int J Cancer* 1993; **54**: 983–986.
33. Fassina G, Aluigi MG, Gentleman S, Wong P, Cai T, Albini A, Noonan DM. The cAMP analog 8-Cl-cAMP

inhibits growth and induces differentiation and apoptosis in retinoblastoma cells. *Int J Cancer* 1997; **72**(6): 1088–1094.

34. Bianco C, Tortora G, Baldassarre G, Caputo R, Fontanini G, Chine S, Bianco AR, Ciardello F. 8-chloro-cyclic AMP inhibits autocrine and angiogenic growth factor production in human colorectal and breast cancer. *Clin Cancer Res* 1997; **3**(3): 439–448.
35. Yu M, Sato H, Seiki M, Spiegel S, Thompson EW. Elevated cyclic AMP suppresses ConA-induced MT1-MMP expression in MDA-MB-231 human breast cancer cells. *Clin Exp Metastasis* 1998; **16**(2): 185–191.
36. Castaigne S, Chomienne C, Daniel MT, Ballerini P, Berger R, Fenaux P, Degos L. All-*trans*-retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. *Blood* 1990; **76**: 1704–1709.
37. Warrell RP Jr, Frankel SR, Miller WH Jr, Scheinberg DA, Itri LM. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans retinoic acid). *N Engl J Med* 1991; **324**: 1385–1393.
38. Makower D, Malik U, Novik Y, Wiernik PH. Therapeutic efficacy of theophylline in chronic lymphocytic leukemia. *Med Oncol* 1999; **16**: 69–71.
39. Thacher SM, Rice RH. Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. *Cell* 1985; **40**: 685–695.
40. Benedetti L, Grignani F, Scicchitano BM, Jetten AM, Diverio D, Lo Coco F, Avisati G, Gambacorti-Passerini C, Adamo S, Levin AA, Pelicci PG, Nervi C. Retinoid-induced differentiation of acute promyelocytic leukemia involves PML-RAalpha-mediated increase of type II transglutaminase. *Blood* 1996; **87**(5): 1939–1950.
41. Arani I, Adler-Storthz K, Chen Z, Tyring SK, Brysk H, Brysk MM. Differentiation markers in oral carcinoma cell lines and tumors. *Anticancer Res* 1997; **17**(6D): 4607–4610.
42. Fesus L. Biochemical events in naturally occurring forms of cell death. *FEBS Lett* 1993; **328**(1–2): 1–5.
43. Autuori F, Farrace MG, Oliverio S, Piredda L, Piacentini M. 'Tissue' transglutaminase and apoptosis. *Adv Biochem Eng Biotechnol* 1998; **62**: 129–136.

(Received 7 March 2000; accepted in revised form 3 May 2000)