PERGAMON

European Journal of Cancer 36 (2000) 1572–1577

European Journal of Cancer

www.ejconline.com

Evaluation of the efficacy of potential antineoplastic drugs on tumour metastasis by a computer-assisted image analysis

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Received 15 November 1999; received in revised form 15 March 2000; accepted 23 March 2000

Abstract

Computerised image analysis, performed on histological sections of (C57BL6/N) mouse lungs that had been intravenously (i.v.) injected with B16-F10 melanoma cells was used to develop a novel method to quantify the efficacy of potential antineoplastic drugs. This procedure allowed the evaluation of the rate of inhibition of growth and the anti-invasive capability of new molecules, thus resulting in more accurate data than that obtained from common macroscopical counting of surface metastatic foci. Several morphological parameters can be measured by this method: the percentage of tissue area occupied by metastases, which accounts for tumour implantation into the organ; the growth index, related to the size of the metastases, and the invasion index, related to the frequency of foci. These morphometric data were found to be correlated to the levels of lung hydroxyproline and transglutaminase activity, well known markers of tumour invasion and cell differentiation, respectively. The main objective of this computerised procedure was to evaluate how the tumour cell is affected in the host by the drug under investigation. The use of the method is exemplified by an analysis of the antitumour activity of some methylxanthines. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Melanoma; Metastasis; Image analysis; Tumour growth; Antineoplastic drugs

1. Introduction

The metastatic spread of cancer cells to different organs represents the major cause of death in cancer patients. Despite the advances recently achieved in the treatment of tumours, the management of malignancies still remains a difficult task for the oncologist [1,2]. Indeed, one of the aims of most cancer researchers is the development of a suitable technical model which could allow both the evaluation of the metastatic potential of tumour cells, and the antineoplastic effect of new molecules.

Computer-assisted image analysis is currently used for the quantification of results for a wide range of biological experimental procedures [3–7]. In the present study, this technique coupled with a valid experimental metastasis model, like lung colony formations following intravenous (i.v.) injection of B16-F10 melanoma cells into syngeneic mice, has been shown to be a simple and reliable tool for a more accurate analysis of lung colonies formed by tumour cells. Moreover, the main advantage of this method is the possibility of evaluating how the antineoplastic activity of new drugs is exerted *in vivo*, discriminating between inhibition of tumour growth and anti-invasive properties. This information may be very useful to investigate whether the effects of the antineoplastic molecule could be quantified and manipulated selectively, in the hope of establishing more targeted anticancer therapeutical interventions.

We have previously shown that a set of morphometric parameters can be measured on histological sections of mouse target organs that have been colonised by B16-F10 melanoma cells [8]. In this report, we describe a method which facilitates the interpretation of these parameters for the objective and quantitative *in vivo* evaluation of invasion and tumour growth for pharmacological studies.

2. Materials and methods

2.1. Materials

Theophylline, caffeine and theobromine were purchased from Sigma (St Louis, MO, USA). [^{3}H]-putrescine 2

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PII: S0959-8049(00)00147-7

HCl (38.15 Ci/mmol) was purchased from NEN (Boston, MA, USA). Culture medium (Dulbecco's minimum essential medium, DMEM), fetal bovine serum (FBS) and 0.5% trypsin/0.1% ethylenediamine tetraacetic acid (EDTA) were from GIBCO Laboratories (Grand Island, NY, USA). All other chemicals were provided by Merck (Darmstadt, Germany).

2.2. Cell culture

B16-F10, highly metastatic murine melanoma cells (obtained from I.J. Fidler, University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA), were propagated under standard culture conditions [9]. Cultures were found to be free from mycoplasma species using the Hoechst staining procedure. The invasive potential has been assessed in an *in vitro* invasion assay, as previously described [10]. The metastatic activity, both spontaneous and experimental, was found to be stable over the time of the experimental procedures. Cell viability was tested by the trypan blue (0.25%) exclusion test at different times of culturing. Cells were treated with methylxanthines (theophylline, caffeine or theobromine) at a final concentration of 1 mM and incubated for 48 h in the same culture conditions (5% CO_2, 37°C). Cells were washed twice with DMEM, harvested with trypsin/EDTA solution, resuspended as isolated cells, counted with a Thoma slide and used for the *in vivo* experiments.

2.3. Animal experiments

Male C57BL6/N mice (6 to 8 weeks old) were obtained from IFFA Credo (L'Abreole, France). Lung metastases were produced by intravenous injection of 2×10^5 viable B16-F10 cells (control), or cells pretreated with methylxanthines suspended in 0.2 ml DMEM into anaesthetised mice (Farmotal, 50 mg/kg body weight). As a control, a group of animals (normal) were injected with 0.2 ml DMEM only. The viability of the injected cells, controlled by trypan blue dye exclusion, was always higher than 95%. For each experimental condition, 8–10 mice were inoculated in the lateral tail vein. Mice were sacrificed by cervical dislocation on the eighteenth day after tumour cell injection. Lungs were rapidly excised, rinsed in phosphate buffered saline (PBS), weighed and further processed. All experimental protocols have been carried out following the *Guidelines for the Welfare of Animals in Experimental Neoplasia* [11] and the ECC Council Directive 86/609, OJL 358, 1 December 1987.

2.4. Hydroxyproline content and transglutaminase activity in mice lung homogenates

The tissue to be analysed was frozen in liquid nitrogen upon excision and stored at −70°C. The lung hydroxyproline content was estimated according to a published procedure [12]. Transglutaminase activity in the lung homogenate was assayed as reported by Chung and Folk [13].

2.5. Histology

Samples were fixed in 10% formalin for 48 h, dehydrated in ethanol and embedded in paraffin. Tissue 10 μm-sized serial sections performed every 100 μm were obtained with an ultramicrotome Leitz 1512 (Germany) and were stained with haematoxylin and eosin.

2.6. Quantitative analysis of the tumours

The number of surface metastases was evaluated by two independent operators. A total of 500 lung foci was considered as the maximum value, and expressed as >500. The precise localisation of B16 melanoma colonies in the lungs and the morphometric evaluation were performed on approximately 100 random lung sections in each lung. Quantification of the portion of lung section occupied by metastatic tissue (percentage of implantation), the growth and invasion indexes were obtained using an integrated image analysis system (Quantimet 970, Cambridge Instruments, UK). Densitometric analysis of digitalised microscopical images were used to discriminate metastases of B16-F10 melanoma from normal lung tissue. The following parameters were measured in each lung section: the percentage of tumour implantation, calculated as the ratio between the total metastatic area (TMA) and the total area of the histological section (TA): % tumour implantation $= \frac{\text{TMA}}{\text{TA}} \times 100$, the growth index (GI), expressed as the ratio between the average area of metastatic foci (AAMF) and the total area of the histological section (TA): $\text{GI} = \frac{\text{AAMF}}{\text{TA}}$, the invasion index (II), calculated as the ratio between the total metastatic area (TMA) and the average area of metastatic foci (AAMF): $\text{II} = \frac{\text{TMA}}{\text{AAMF}}$.

2.7. Statistical analysis

Values for tumour-bearing mice injected with untreated B16 cells were compared with those for mice injected with methylxanthine-treated cells. The results were expressed as the mean±standard error of the mean (SEM) of the values obtained for each lung. All experimental data were analysed by non-parametric Mann–Whitney test (P values lower than 0.05 were considered as significant). Statistical correlations between morphometric parameters (GI and II) and biochemical data (transglutaminase activity and hydroxyproline content, respectively), have been carried out by Bravais–Pearson correlation test (P values lower than 0.05 were considered as significant).

3. Results

The effect of methylxanthine treatment of B16-F10 melanoma cells on hydroxyproline content and transglutaminase activity in the lung homogenate is shown in Table 1. Hydroxyproline content was studied as a marker of lung invasion of the tumour cells [14,15]. In control tumour-bearing mice, lung hydroxyproline content was 12.5±1.0 μg/mg protein. Treating tumour cells with 1 mM theophylline or caffeine, this value significantly decreased to 6.4±0.8 μg/mg protein ($P < 0.001$) and to 10.0±1.2 μg/mg protein ($P < 0.005$), respectively. Theobromine did not influence hydroxyproline levels (11.2±1.3 μg/mg protein). Transglutaminase activity, considered as a differentiation marker [16], in control tumour-bearing mice was found unchanged from normal animals (1.56±0.14 pmol [^{3}H]-putrescine/h/mg protein), and significantly increased with theophylline treatment (15.43±1.23 pmol [^{3}H]-putrescine/h/mg protein, $P < 0.001$). A lesser increase in transglutaminase activity was found in the caffeine-treated group (4.67±0.24 pmol [^{3}H]-putrescine/h/mg protein, $P < 0.001$), whereas the value for the theobromine group was similar to the control (1.76±0.15 pmol [^{3}H]-putrescine/h/mg protein).

Fig. 1 shows the appearance of the mouse lungs three weeks after the inoculation of melanoma cells. As usual, the quantitative estimation of the antineoplastic activity of the drug under investigation was done by counting the number of surface metastases. Fig. 2 shows that theobromine-treated cells (Tb) produced values similar to the control (Ctrl > 500 foci/lung), whereas tumour implantation appeared lower (44%) for caffeine-treated cells (Cf = 282±22 foci/lung, $P < 0.005$) and particularly reduced (72%) for theophylline-treated cells (Th = 138±12 foci/lung, $P < 0.001$). The histological section of lungs invaded by untreated or theophylline-treated B16 cells, showed that the melanoma colonies were usually located either inside (Mi) or superficially (Ms) near pulmonary blood vessels and bronchioles

Table 1
Determination of hydroxyproline content and transglutaminase activity in lungs from C57BL6/N mice intravenously (i.v.) injected with untreated B16-F10 melanoma cells (control) or with cells treated with 1 mM theophylline, caffeine or theobromine

Treatment	Lung hydroxyproline (μg/mg protein)	Lung transglutaminase activity (pmol [^{3}H]-putrescine/h/mg protein)
Normal[a]	0.8±0.05	1.41±0.12
Control	12.5±1.0	1.56±0.14
Theophylline	6.4±0.8[b]	15.43±1.23[b]
Caffeine	10.0±1.2[c]	4.67±0.24[b]
Theobromine	11.2±1.3	1.76±0.15

[a] Injected with vehicle only.
[b] Significantly different from control, $P < 0.001$.
[c] Significantly different from control, $P < 0.005$.

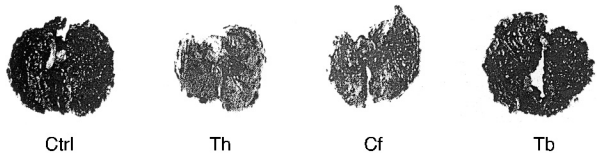

Fig. 1. Representative lungs of C57BL6/N mice intravenously (i.v.) injected with untreated B16-F10 melanoma cells (Ctrl) or treated for 48 h with 1 mM theophylline (Th), caffeine (Cf) or theobromine (Tb). Superficial metastases are visible as black spots. Approximately 20 mice were treated for each experimental group.

(Fig. 3). The area of lung tissue occupied by metastases was calculated on each histological section by means of a computerised procedure. Fig. 4 shows the percentage of implantation of melanoma cells into the target organ. Theophylline treatment of melanoma cells inhibits tumour implantation more than caffeine (51% for theophylline, $P < 0.005$; 24% for caffeine, $P < 0.005$), whereas theobromine did not exert any effect. Higher magnification of the histological sections of the mouse lungs is shown in Fig. 5. In the control experiments (Ctrl), the lung foci (arrowed) appeared larger than those obtained with theophylline-treated tumour cells (Th), and metastases were also visible in contact with pleura of the organ. Caffeine-treated melanoma cells (Cf) produced lung foci which appeared reduced with respect to the control, whereas in the lung sections obtained from mice injected with theobromine-treated cells (Tb), negligible variations in tumour colonisation were observed.

In order to give a measure of the rate of tumour growth in the target organ, expressed as the growth index (GI), and of the frequency of foci in the lung,

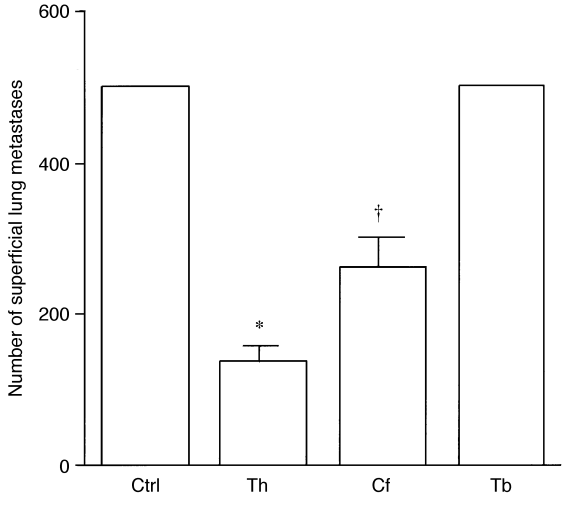

Fig. 2. Quantitative evaluation of the number of superficial lung metastases from melanoma cells intravenously (i.v.) injected into C57BL6/N mice. Ctrl, untreated cells; Th, cells treated with 1 mM theophylline; Cf, cells treated with 1 mM caffeine; Tb, cells treated with 1 mM theobromine. Each group represent the mean±SEM of approximately 20 mice. The counting of lung foci was limited to a maximum of 500. *$P < 0.001$; †$P < 0.005$.

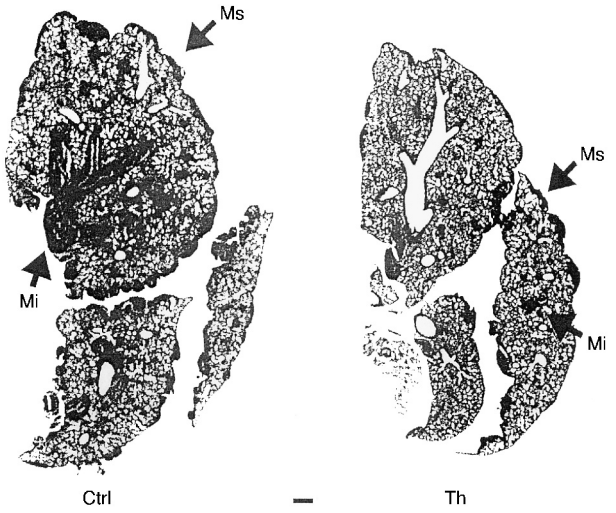

Fig. 3. Histological sections of lung from C57BL6/N mice intravenously (i.v.) injected with untreated B16-F10 melanoma cells (Ctrl) or treated for 48 h with 1 mM theophylline (Th). Scale bar: 0.2 mm. Mi, internal metastases; Ms, superficial metastases.

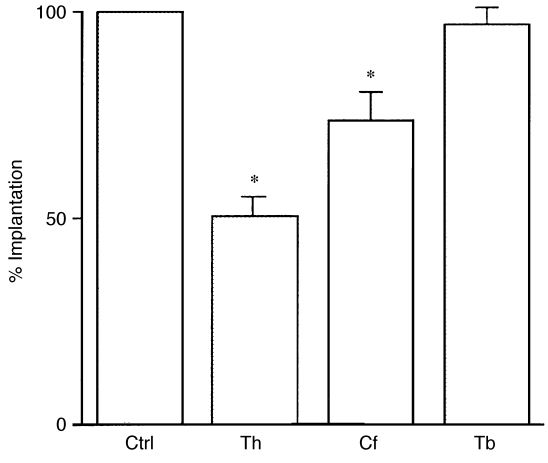

Fig. 4. Quantitative evaluation by computer-assisted image analysis of the percentage of lung colonisation by B16-F10 melanoma cells intravenously (i.v.) injected into C57BL6/N mice, performed on histological sections of lung. The percentage of implantation represents the ratio between the total metastatic area and the total area of the histological section. Ctrl, untreated cells; Th, cells treated with 1 mM theophylline; Cf, cells treated with 1 mM caffeine; Tb, cells treated with 1 mM theobromine. Each group represents the mean±SEM of approximately 20 mice. $*P < 0.005$.

expressed as the invasion index (II), all the histological sections obtained from B16-F10 colonised lungs were further analysed by a computer-assisted image analysis system (Quantimet). The values of GI and II are shown in Table 2. The magnitude of the two indexes was not affected by the treatment of B16 cells with theobromine ($GI = 156 \pm 12 \times 10^{-4}$; $II = 389.1 \pm 22.3$) with respect to the control ($GI = 160 \pm 10 \times 10^{-4}$; $II = 369.7 \pm 21.7$). In contrast, the indexes for theophylline and caffeine were significantly reduced. From these data, it was also possible to observe that, while the reduction of tumour growth by theophylline ($GI = 48 \pm 3 \times 10^{-4}$, $P < 0.001$) and caffeine ($GI = 62 \pm 5 \times 10^{-4}$, $P < 0.001$) is similar, theophylline exerts an higher anti-invasive capability ($II = 105.8 \pm 17.7$, $P < 0.001$) than caffeine ($II = 278.2 \pm 16.4$, $P < 0.005$). Table 2 reports the statistical correlations between the two morphometric indexes and the biochemical parameters shown in Table 1. The values of r (Bravais-Pearson correlation coefficient) appear highly significant for GI/transglutaminase activity (negative correlation), and II/hydroxyproline levels (positive correlation).

4. Discussion

The adhesion of tumour cells to the endothelial plasma membrane and to the basement membrane proteins, followed by the secretion of specific metalloproteinases, allow metastatic cells to invade tissue and then to proliferate into the target organ [1,17,18]. This complex process is essential for the formation of metastases and is, therefore, of critical clinical importance. For detailed investigations of the antineoplastic effect of new molecules, quantifiable *in vivo* models of tumorigenesis are necessary. In this study, we describe an image analysis procedure which facilitates an objective analysis of experiments carried out using the melanoma cell line B16-F10 in an experimental metastasis model. This procedure appears useful for the *in vivo* investigation of potential antineoplastic molecules.

Table 2
Morphometric parameters obtained by computer-assisted image analysis performed on histological sections of B16-F10 invaded mice lungs and their correlation with transglutaminase activity and hydroxyproline levels

Treatment	Growth index (GI) ($\times 10^4$)	Correlation with transglutaminase activity (r)	Invasion index (II)	Correlation with hydroxyproline levels (r)
Control	160±10	−0.903 ($n = 10$; $P < 0.01$)	369.7±21.7	0.948 ($n = 10$; $P < 0.01$)
1 mM theophylline	48±3[a]	−0.876 ($n = 10$; $P < 0.01$)	105.8±17.7[a]	0.919 ($n = 10$; $P < 0.01$)
1 mM caffeine	62±5[a]	−0.885 ($n = 10$; $P < 0.01$)	278.2±16.4[b]	0.650 ($n = 10$; $P < 0.05$)
1 mM theobromine	156±12	−0.959 ($n = 10$; $P < 0.01$)	389.1±22.3	0.959 ($n = 10$; $P < 0.01$)

r = Bravais-Pearson correlation coefficient.
[a] Significantly different from control, $P < 0.001$.
[b] Significantly different from control, $P < 0.005$.

Since the extent of the area of a metastatic focus is directly related to tumour growth, we considered as growth index (GI) the ratio between the average area of metastatic foci (AAMF) over the total area of the histological sections (TA). Furthermore, assuming that a lung focus is evolved from at least a single tumour cell, which migrated through the basement membrane and invaded the target tissue, the number of lung foci is roughly related to the invasion of tumour cells, the parameter indicated as Invasion Index (II = TAM/AAMF). The values found for these two morphometric parameters, appear to be supported by the biochemical data obtained in the target organ invaded by the melanoma cells. Indeed, it appears that the GI negatively correlates with a differentiation marker (transglutaminase activity), whereas the II positively correlates with an invasion marker (hydroxyproline content). These findings strongly suggest that our morphometric procedure provides a reliable evaluation of the *in vivo* tumour growth and invasion rates, highlighting its potential utility in the investigation of the antineoplastic capability of new chemical agents.

Since promising preclinical results have been reported for theophylline [19], we tested our method using methylxanthines with variable antineoplastic activity [8,20]. Histopathological analysis of lung tissue showed infiltration by neoplastic cells around the main bronchioles and extending to the pleura in the control tumour-bearing animals (Fig. 5, Ctrl). Pretreating B16-F10 cells with theophylline or caffeine, the tumour foci were localised more often internally in the organs, thus suggesting a preferential arrest of malignant cells in larger vessels rather than in the peripheral capillary bed. This observation may explain the discrepancy between data obtained counting the lung surface metastatic foci (Fig. 2) and our estimation on the histological sections of the target organ, which also includes the internal foci (Fig. 4). Indeed, the antineoplastic activity of theophylline appears overestimated by the surface counting of melanoma foci (72% reduction), when compared with Fig. 4 (51% reduction). The results obtained by our digitalised procedure, supported by the biochemical markers, demonstrated that theophylline affected the tumour implantation more than caffeine and theobromine treatments. For theophylline, the antineoplastic activity appears to be exerted by the impairment of tumour growth and cell invasiveness, as shown by the increase of transglutaminase activity, which led to cell differentiation [16,21], and by the decrease of hydroxyproline content (Table 1). This is supported by the lowered GI and II observed for this treatment (Table 2). On the contrary, as shown in Table 2, the antineoplastic activity

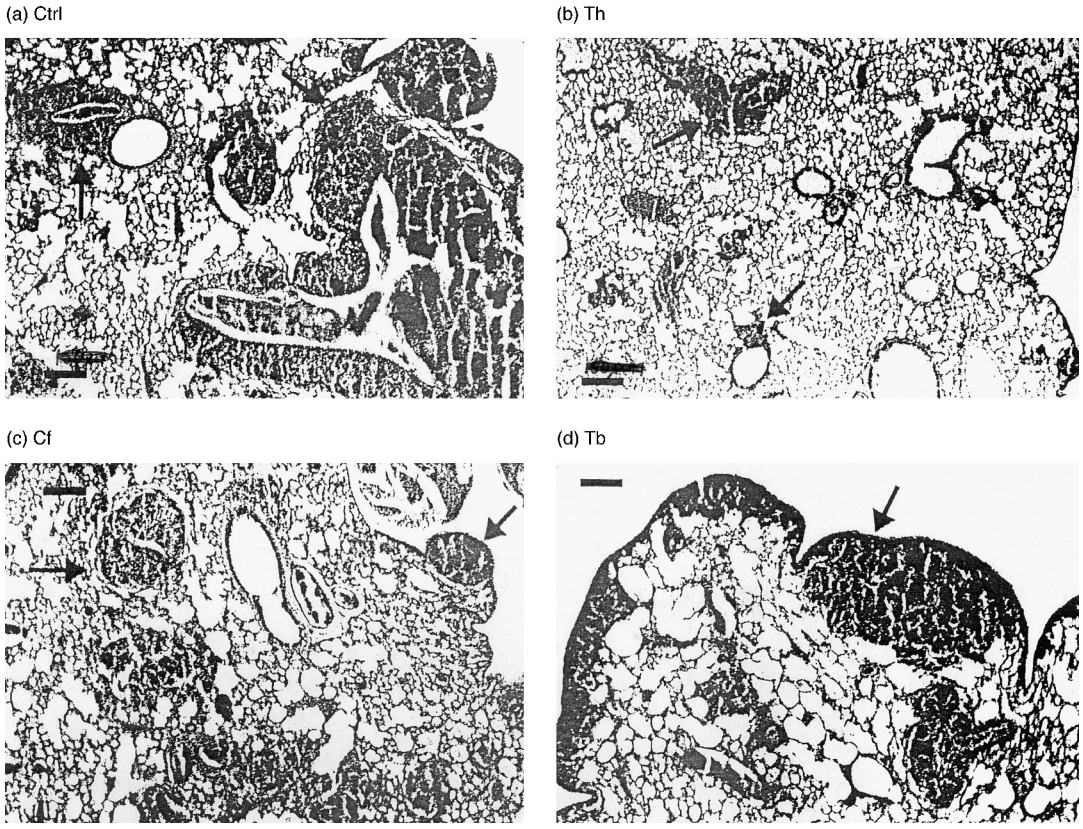

Fig. 5. Histological sections of lung of C57BL6/N mice intravenously (i.v.) injected with untreated B16-F10 melanoma cells (Ctrl) or treated for 48 h with 1 mM theophylline (Th), caffeine (Cf) or theobromine (Tb). Arrows indicate melanoma metastases inside the parenchymal tissue. Scale bars (Ctrl, Th, Cf, Tb): 150 μm.

caused by caffeine appears ascribed to a greater influence on tumour growth than on cell invasiveness. These findings highlight a further advantage of this method, the possibility of the *in vivo* discrimination between inhibition of tumour growth and the anti-invasive capability of the drug under investigation. Moreover, the data presented here provide strong direct evidence that a detailed image analysis of the metastatic foci in histological lung tissue sections is much more precise than a simple macroscopical surface counting. The macroscopical evaluation is in fact usually based only on the visualisation of superficial tumours of relatively large size. This is particularly evident considering that the rate of pigmentation, localisation of foci and size of the individual tumours are independent parameters which, as documented here, can be critically modulated by treatment with different compounds. When the effects of different methylxanthines were investigated microscopically, the influence on the distribution, size and number of melanoma colonies in the lungs could be more precisely calculated. On the basis of these considerations, we propose that the combination of a computerised image analysis and an experimental metastatic model of B16-F10 melanoma cells might be a valuable research tool for studying new therapeutic drugs for cancer.

Acknowledgements

We would like to thank Dr Francesco Facchiano for helpful suggestions and Professor E. Capucci (University of Rome 'Tor Vergata') for the statistical analysis. This work was supported by grants from Consiglio Nazionale delle Ricerche (Target Project 'Invecchiamento' n. 9200339) and from the University of Rome 'Tor Vergata' (Project 'Giovani Ricercatori' n. 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, MA, Martinus Nijoff, 1984, 15–38.
3. Suh O, Weiss L. The development of a technique for the morphometric analysis of invasion in cancer. *J Theor Biol* 1984, **108**, 547–562.
4. Kostenuik PJ, Singh G, Suyama KL, Orr W. A quantitative model for spontaneous bone metastasis: evidence for a mitogenic effect of bone on Walker 256 cancer cells. *Clin Exp Metastasis* 1992, **10**, 403–410.
5. Coucke PHA, Lambertz M, Baramowa EN, *et al*. Re-evaluation by image analysis of the effects of fibroblasts, fibronectin or laminin upon colony formation in mouse lungs by B16 melanoma cells. *Invasion Metastasis* 1993, **13**, 201–211.
6. Belien JA, Somi S, De Jong JS, Van Diest PJ, Baak JP. Fully automated microvessel counting and hot spot selection by image processing of whole tumour sections in invasive breast cancer. *J Clin Pathol* 1999, **52**, 184–192.
7. Landau M, Matz H, Tur E, Dvir M, Brenner S. Computerized system to enhance the clinical diagnosis of pigmented cutaneous malignancies. *Int J Dermatol* 1999, **38**, 443–446.
8. Lentini A, Kleinman HK, Mattioli P, *et al*. Inhibition of melanoma pulmonary metastasis by methylxanthines due to decreased invasion and proliferation. *Melanoma Res* 1998, **8**, 131–137.
9. Fidler IJ. Selection of successive tumor lines for metastases. *Nature (New Biol)* 1973, **245**, 148–149.
10. 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.
11. Guidelines for the Welfare of Animals in Experimental Neoplasia. *Br J Cancer* 1998, **77**, 1–10.
12. Bergman I, Loxley R. The determination of hydroxyproline in urine hydrolysates. *Clin Chim Acta* 1970, **27**, 347–349.
13. Chung SI, Folk JE. Transglutaminase from hair follicle of guinea pig. *Proc Natl Acad Sci (Wash)* 1972, **69**, 303–307.
14. Liotta LA, Kleinerman J, Catanzaro P, Rynbrandt D. Degradation of basement membrane by murine tumor cells. *J Natl Cancer Inst* 1977, **58**, 1427–1431.
15. Barsky SH, Gopalakrishna R. An experimental model for studying the desmoplastic response to tumor invasion. *Cancer Lett* 1987, **35**, 271–279.
16. Rice RH, Mehrpouyan M, O'Callahan W, Parenteau NL, Rubin AL. Keratinocyte transglutaminase: differentiation marker and member of an extended family. *Epithelial Cell Biol* 1992, **1**, 128–137.
17. Liotta LA, Rao CN, Barsky SH. Tumor invasion and the extracellular matrix. *Lab Invest* 1983, **49**, 636–649.
18. Nicolson GL. Cell surface molecules and tumor metastases. *Exp Cell Res* 1984, **150**, 3–22.
19. Makower D, Malik U, Novik Y, Wiernik PH. Therapeutic efficacy of theophyline in chronic lymphocytic leukemia. *Med Oncol* 1999, **16**, 69–71.
20. 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**, 274–278.
21. 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.