Pergamon

European Journal of Cancer, Vol. 35, No. 10, pp. 1526–1533, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00176-8

Original Paper

Low-Doses of Ionising Radiation Induce Melanoma Metastases and Trigger the Immune System—Adrenal Axis Feedback Loop

E.M. Link,[1] K. Flanagan,[1] A.S. Michalowski[1] and P.J. Blower[2]

[1]Department of Molecular Pathology, University College London Medical School, Windeyer Institute, 46, Cleveland Street, London W1P 6DB; and [2]Department of Nuclear Medicine, Kent and Canterbury Hospital, Canterbury, U.K.

Low-doses of ionising radiation are frequently implicated in triggering and/or accelerating the growth of skin and other malignancies. It seemed probable that the radiation at similar dose levels might initiate metastasis from already existing tumours. Highly pigmented human melanoma xenograft that had lost its ability for a spontaneous metastasising and grown subcutaneously in athymic mice was exposed to very low and well-defined doses of ionising radiation to determine whether low linear energy transfer radiation can restore metastatic potential of the tumour. To ensure that all effects derived from radiation-activated neoplastic cells only, ^{131}I was delivered selectively to the cutaneous melanoma instead of using the external beam. The direct response of these tumours to radiation was monitored by determining the growth rate of the lesions. Histopathological methods were employed to detect metastases. The lowest radiation dose of approximately 6 cGy deposited in the tumours initiated metastatic spread in all animals. Gradual increase of the radiation doses diminished both the frequency of the appearance of metastases and their distance from the primary lesions. There were no metastases from non-irradiated melanomas. The highest dose used (60 cGy) did not affect significantly the growth of cutaneous (primary) tumours, but lower doses that enhanced inflammatory infiltration of the lesions reduced tumour growth. Such radiation-stimulated immune responses were accompanied by increased pigmentation in cutaneous lesions and activation of the adrenal cortex indicating that the immune system–adrenal axis feedback loop had been triggered. The results demonstrate that very low-doses of ionising radiation induce melanoma metastases. The phenomenon is accompanied by the stimulation of the immune system–adrenal axis feedback loop that regulates eicosanoid synthesis, thereby suggesting an involvement of these molecules in the process. Radiation doses approaching the therapeutic level do not initiate melanoma dissemination. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: melanoma, metastases, ionising radiation, immune system, inflammation, immune system—adrenal axis feedback loop, hydrogen peroxide, eicosanoids
Eur J Cancer, Vol. 35, No. 10, pp. 1526–1533, 1999

INTRODUCTION

MELANOMAS WITH a thickness of only 1 mm metastasise with a probability of almost 100% [1]. This exceptionally high potential of melanoma cells to form local (satellite) and distant lesions places the neoplasm among therapeutically most difficult tumours. At the same time it makes melanoma a very attractive model for studying the phenomenon.

The 'physiological' factors triggering the metastatic cascade remain unidentified. However, environmental involvement such as ultraviolet (UV) radiation is now commonly accepted as a potent contributor to both the initiation of melanoma growth and its subsequent metastatic spread [2–4]. The mechanism of UV-mediated acceleration of the process is, however, obscure. Similarly, although the mechanism

Correspondence to E.M. Link, e-mail: e.link@ucl.ac.uk
Received 4 Jan. 1999; revised 27 May 1999; accepted 8 Jun. 1999.

is still under investigation [5], carcinogenic properties of low-doses of ionising radiation, as well as the increased appearance of some forms of cancer among individuals exposed to such doses in the workplace or due to environmental hazards are already well documented [6, 7]. The possibility of stimulation or even initiation of the metastatic process in tumours irradiated *in vivo* with low-doses of ionising radiation is less appreciated, although there are some indications that the low exposure of the existing lesions to ionising radiation may enhance metastasis, and the preferential localisation of the secondaries derived from non-irradiated tumours is in the previously irradiated tissues reviewed in [8].

Since frequent exposure to very low doses of ionising radiation is an every-day experience of a significant percentage of the population and various forms of radiation are commonly applied in several diagnostic procedures, it seems of great importance to elucidate whether ionising radiation at this low-dose range might trigger metastatic dissemination from the already existing tumours and, if this is the case, to determine the mechanism involved in the process.

The high metastatic capacity of melanoma favours the use of this tumour for investigating the phenomenon. A human melanoma xenograft that has lost its ability for spontaneous metastasising in mice represents an attractive model to study whether low radiation doses can restore metastatic potential of the xenograft. The aim of this investigation was to test the ability of ionising radiation to re-initiate the metastatic process using this model.

MATERIALS AND METHODS

Experimental tumour and its growth in vivo

A subline of HX118 human melanoma xenograft that had gradually lost its ability to spontaneously metastasise was used in the study. The original xenograft derived from a biopsy sample of the secondary lymph node deposit of a patient who had not undergone cytotoxic therapy prior to the biopsy (the tissue was provided by courtesy of G.G. Steel of the Institute for Cancer Research, Sutton, U.K. after being established by J. Mills of the same institute).

The material used for subsequent experiments was derived from inguinal tumours passaged *in vivo* in 50–60-day-old athymic female mice lacking T cells only ([CRL:nu-nu (CD/1^{TM})BR] supplied by Charles River, U.K.) every 5–6 weeks. Small tumour fragments from HX118 xenografts were suspended in Eagle's minimum essential medium (MEM; from Flow Laboratories, Ltd, Irvine, U.K.) and inoculated subcutaneously into recipient mice (three approximately 1 mm^2 pieces suspended in 0.2 ml medium per site). Every experimental animal presented two cutaneous inguinal lesions.

Radiation-mediated induction of metastases

Induction of metastases was attempted by applying low doses of ionising radiation to the primary lesions. To ensure that all effects derived from radiation-activated melanoma cells rather than from normal irradiated tissues, ^{131}I delivered selectively to the cutaneous lesions was used instead of the external beam. (Methylene blue (MTB) with its high affinity to melanin employed as a carrier for ^{131}I secured a selective uptake of the radioisotope by melanoma cells [9–14]. High radiation doses delivered to melanoma cells by MTB labelled with the properly chosen radioisotope effectively inhibit the growth of the tumour regardless of its size and localisation [15–19]. Cold MTB (that is, without a radioisotope) does not affect melanoma and normal tissues at the concentrations employed [10, 11, 13–19].)

Forty-six athymic mice bearing human pigmented HX118 melanoma xenografts (two tumours per animal) were used for the experiments. The animals were divided into five groups: the control mice were given either cold MTB or none (total of 16 animals in three independent experiments) and four experimental groups where mice were injected with 0.17 MBq (total of 12 animals in three independent experiments), 0.6 MBq (total of six mice in two independent experiments), 1.4 MBq (total of four animals in a single experiment) and 1.7 MBq of ^{131}I-MTB (total of eight mice in two independent experiments). The corresponding radiation doses deposited in the tumours amounted to approximately 6, 21, 49 and 60 cGy, respectively. The radioisotope was administered to mice intravenously (i.v.) on day 14, that is when the cutaneous lesions reached an average size of 3.9 mm×2.7 mm×1.3 mm (the greatest diameter, perpendicular to it and the thickness, respectively) to match the 2 mm range of β-electrons emitted by ^{131}I.

Detection of metastases: microscopic analysis

Animals injected with ^{131}I-MTB were culled under halothane general anaesthesia between days 70 and 90 except for the majority of animals injected with 1.4 and 1.7 MBq of ^{131}I-MTB and observed for up to 153 days owing to a delay in the growth of irradiated cutaneous lesions. The average size of tumours at the time of culling was 17.6 mm×13.5 mm×9.1 mm. The tumours, heart, lungs, liver, kidneys, spleen, lymph nodes from the site of the xenografts and adrenal glands were excised, fixed with 10% formal saline and prepared for light microscopic examination following standard methods of paraffin wax embedding, cutting and staining with haematoxylin and eosin [20]. Several specimens chosen randomly from every organ were analysed in detail [17, 19]. The number of specimens taken from each organ was always the same and serial sectioning was performed on every specimen.

Determination of growth rate of primary tumour

The rate of growth of cutaneous tumours was determined by calculating a ratio of tumour size at time intervals after their inoculation to the size of the tumours 14 days after implantation, that is the time of radioisotope administration. The size of the lesions was estimated as previously described [16, 18, 19], from repeated calliper measurement of three diameters of the tumour: the greatest and the two perpendicular to it. The thickness of the skin in close proximity to the lesion was determined in every mouse and subtracted from the thickness of the tumour.

Statistical analysis and curve fitting

The significance of differences between the growth rate of control and experimental tumours was calculated using the Student *t*-test in which slopes of the relevant curves were compared [21]. The lines in all figures were drawn directly through experimental points using Microsoft Excel.

RESULTS

Microscopic analysis of tumours and normal organs

Control group: Cutaneous primary tumours. All melanoma lesions were highly pigmented and encapsulated. Inflammatory

Table 1. *Radiation-dependent induction of metastases, inflammatory infiltration of the primary tumours and activation of the cortex of the adrenal glands in athymic mice bearing HX*118 *human melanoma xenografts*

Radioactivity injected (MBq)	Presentation of metastases (% animals)	Localisation of metastases	Inflammatory infiltration of cutaneous tumours (% tumours)		Activation of adrenal cortex (% adrenals)	
			At the margin	In the tumour	Glomerulosa	Fasciculata
None	0	–	69 (Residual)	35 (Residual)	76 (Residual)	7 (Residual)
0.17	100	Lungs, bronchus, mammary glands	81	38	100	86
0.60	33	Lymph nodes	9	0	33 (Residual)	33 (Residual)
1.4	25	Connective tissue (satellite to primary tumour)	81 (Extensive)	63 (Extensive)	40	100
1.7	A single lesion	Blood vessel, adjacent to primary tumour	75	44	71	100

cells (lymphocytes and plasma cells) grouped in small foci were observed in the periphery of 69% of tumours, whereas pigment-laden macrophages often accompanied blood vessels incorporated deep into the necrotic tissue (Table 1). Host adipocytes and deposits of calcium salts were also found within these tumours. There was no difference in the appearance of tumours from animals injected with cold MTB and those derived from mice given none.

Metastases. Metastatic lesions, either local or distant, were not found in control animals regardless of whether the mice were injected with cold MTB or given none.

Experimental groups (radiation-stimulated dissemination of melanoma): Cutaneous primary tumours. Cutaneous lesions in all experimental groups (that is, regardless of the radioactivity of administered ^{131}I) were highly but unevenly pigmented and, unlike in control animals, with incomplete encapsulation (50% of lesions). Tumour necrosis increased progressively with the radioactivity of injected ^{131}I and was characterised by a high proportion of pyknotic cells and apoptotic cells within some lesions exposed to 0.17 MBq or liquefaction and haemorrhages after administration of 0.6 MBq of ^{131}I. Extensive angiogenesis was observed in the immediate vicinity of and within the tumours after administration of 0.17 MBq (in 75% of animals) and 1.4 MBq (25% of mice) of ^{131}I (Figure 1). The lesions also incorporated host adipocytes. In those exposed to 1.4 MBq of ^{131}I, host connective tissue was also observed. Host blood vessels embodied deep within the tumour mass were found in the lesions from animals injected with either 1.4 or 1.7 MBq of ^{131}I. More than 70% of tumours exposed to 0.17, 1.4 and 1.7 MBq of ^{131}I presented extensive peripheral inflammatory infiltrate usually arranged in foci surrounding the lesions and consisting of either lymphocytes and granulocytes (0.17 and 1.7 MBq of ^{131}I) or, alternatively, lymphoblasts, lymphocytes (predominantly small) and plasma cells (1.4 MBq of ^{131}I) (Table 1, see also Figure 1a). Additionally, a deep extensive infiltration of small lymphocytes and a significant number of pigment-laden macrophages in these lesions accompanied by the humoral immune reaction was found in animals injected with ^{131}I of the latter radioactivity (Figure 1b).

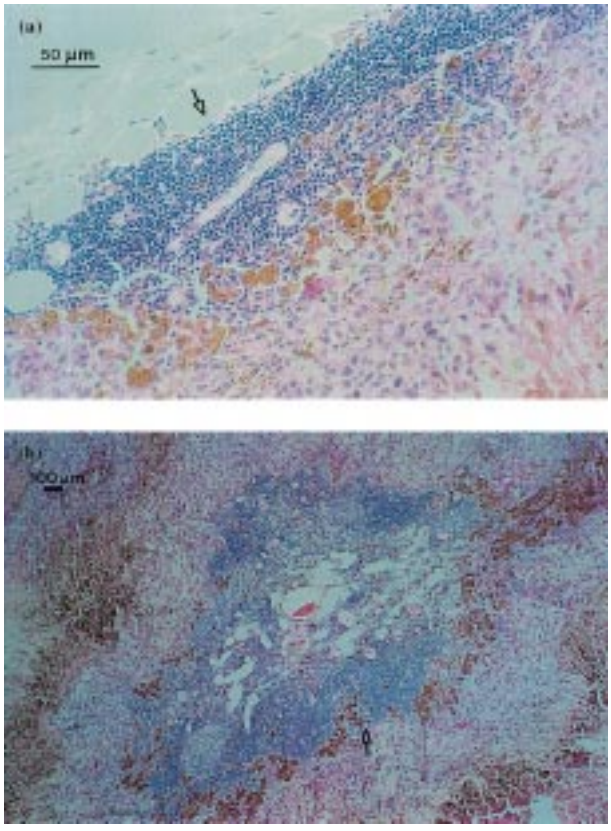

Figure 1. Microscopic appearance of pigmented HX118 human melanoma xenograft inoculated subcutaneously into an athymic mouse and exposed to ^{131}I incorporated within tumour cells after intravenous injection of (a) 0.17 MBq; and (b) 1.4 MBq ^{131}I-MTB. Haematoxylin and eosin staining. (a) Extensive peripheral inflammatory infiltrate (dark blue staining indicated by an arrow) accompanied by angiogenesis and increased pigmentation of cells localised in close proximity to the inflammation site found in 81% of tumours exposed to 6 cGy. Magnification ×100. (b) A deep extensive infiltration of small lymphocytes (dark blue staining indicated by an arrow) surrounded by intensely pigmented cells and with angiogenesis in the centre found in 63% of tumours exposed to the radiation dose of 49 cGy. Magnification ×40.

The only tumours almost free of inflammatory infiltration were those exposed to 0.6 MBq ^{131}I (Table 1). However, pigment-laden macrophages were observed in these lesions.

Metastases. Metastases were induced by all investigated radioactivities of ^{131}I (Table 1). However, their number, frequency of appearance and the distance from the primary tumours were reciprocal to the radioactivity of the administered radionuclide (Table 1). Localisation of secondary lesions varied from the distant, deposited preferentially in lungs (0.17 MBq), to satellite in the vicinity of cutaneous tumours (1.4 MBq).

Metastases (single or multiple) were detected in all animals injected with 0.17 MBq ^{131}I whereas only 33% of mice given 0.6 MBq of the radioisotope presented distant lesions (Table 1, also Figure 2a and b]. Cutaneous tumours exposed to ^{131}I after i.v. injection of 1.4 MBq ^{131}I-MTB were accompanied by small, encapsulated satellite metastases localised in the surrounding connective tissue of 25% of animals (Table 1, Figure 2c). A single satellite deposit invading a blood vessel adjacent to the implantation site of the primary tumour was found in one mouse after administration of 1.7 MBq ^{131}I (Table 1).

Normal organs. The appearance of normal organs was generally the same as in controls. The only difference, as found in approximately 75% of animals injected with 0.17, 1.4 and 1.7 MBq of ^{131}I (but not with 0.6 MBq), was seen in the spleen and lymph nodes that were activated (that is the lymph nodes presented sinusoidal desquamation and trabecular thickening, whereas the spleen presented clearly defined germinal centres). There was also inflammatory infiltration of plasma cells and lymphocytes in the form of small foci associated with the portal spaces of the liver, particularly in close proximity to the biliary ducts and veins in the animals so treated. Animals injected with 1.4 MBq of ^{131}I also revealed an extensive humoral immune reaction.

In addition to the changes observed in the spleen and lymph nodes, a capsular thickening of adrenals and cell proliferation in the zona glomerulosa, as well as the activation of the zona fasciculata of the gland's cortex, often extensive, were found in mice inoculated with 0.17, 1.4 and 1.7 MBq ^{131}I but not so much in those given 0.6 MBq (Table 1, Figure 3]. Whilst the lowest ^{131}I radioactivity used was equally effective in stimulating cell proliferation in the zona glomerulosa and activating the zona fasciculata, the two highest doses of the radioisotope affected predominantly the zona fasciculata with lesser effects on the zona glomerulosa (Table 1).

Growth rate of cutaneous primary tumours—comparison with the lesion's morphological appearance

The growth rate of cutaneous (primary) melanoma was not influenced by cold MTB (Figure 4a). Radiation emitted by ^{131}I deposited within melanoma cells of the cutaneous tumours did not significantly affect the growth of these lesions except at the highest radioactivity used (1.7 MBq) which temporarily diminished the average rate of growth of the majority of tumours in this group by 26–53% as compared with controls ($P = 0.02$ and $P < 0.01$, respectively) (Figure 4e). An incorporation of ^{131}I characterised by the two lowest radioactivities employed was associated with slight but significant acceleration in the growth of some lesions. Such an increase in the growth rate was observed either with a few weeks delay following the administration of the radioisotope (0.17 MBq, $P = 0.02$) (Figure 4b) or immediately after its injection (0.6 MBq, $P = 0.05$) (Figure 4c). Growth of the remaining tumours differed very little from control values. The most heterogeneous response of cutaneous melanoma to

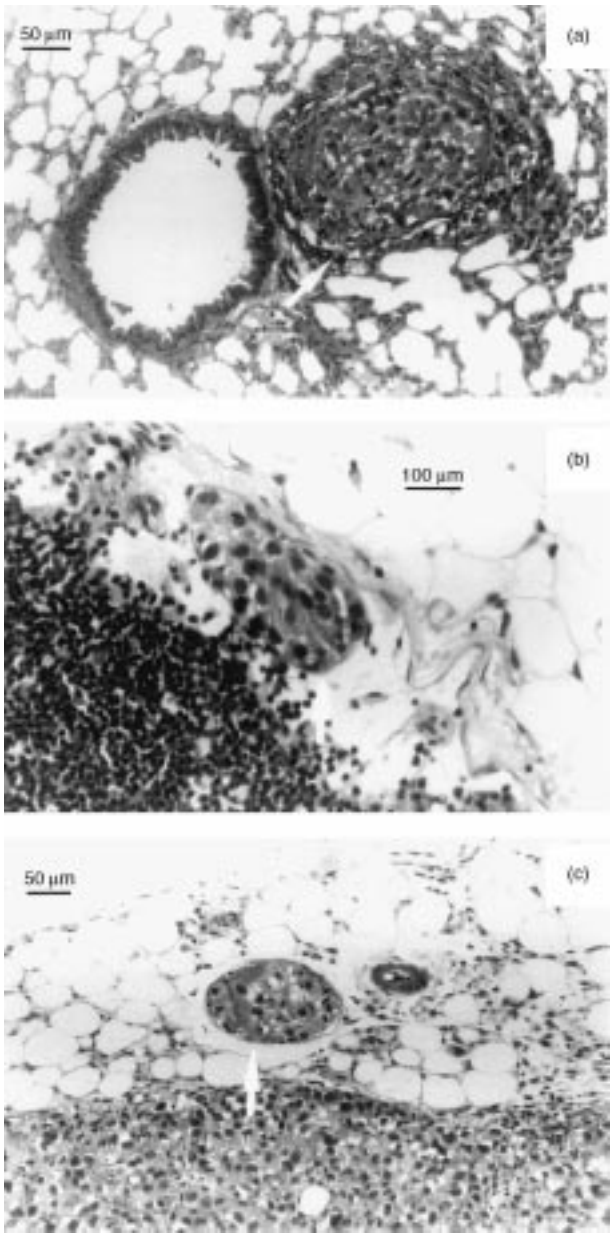

Figure 2. Melanoma metastases derived from the cutaneous primary HX118 tumours with incorporated ^{131}I and grown in athymic mice. Haematoxylin and eosin staining. (a) Well formed metastatic lesion (indicated by an arrow) in close proximity to a small bronchus found after intravenous administration of 0.17 MBq ^{131}I-MTB which delivered a radiation dose of 6 cGy to the primary tumours. Metastases were detected in all animals injected with ^{131}I-MTB characterised by this radioactivity. Magnification ×200. (b) Metastatic lesion (indicated by an arrow) anchored to the wall of a peripheral lymph vessel in a lymph node found after intravenous administration of 0.6 MBq ^{131}I-MTB which delivered a radiation dose of 21 cGy to the primary tumours. Metastases were detected in 33% of animals injected with ^{131}I-MTB characterised by this radioactivity. Magnification ×400. (c) Encapsulated satellite metastasis of a cutaneous primary tumour (indicated by an arrow) found after intravenous administration of 1.4 MBq of ^{131}I-MTB which delivered a radiation dose of 49 cGy. Metastases were found in 25% of animals injected with ^{131}I-MTB characterised by this radioactivity. Magnification ×200.

^{131}I was found in the group of animals inoculated with 1.4 MBq of the radioisotope (Figure 4d). It varied from almost total inhibition of the tumour growth to growth similar to that of control lesions. A diminished growth rate correlated with the extent of the humoral immune reaction and a deep infiltration of lymphoblasts, small lymphocytes, plasmocytes and pigment-laden macrophages in the lesions, as well as the degree of aggressiveness with which they invaded the surrounding tissue.

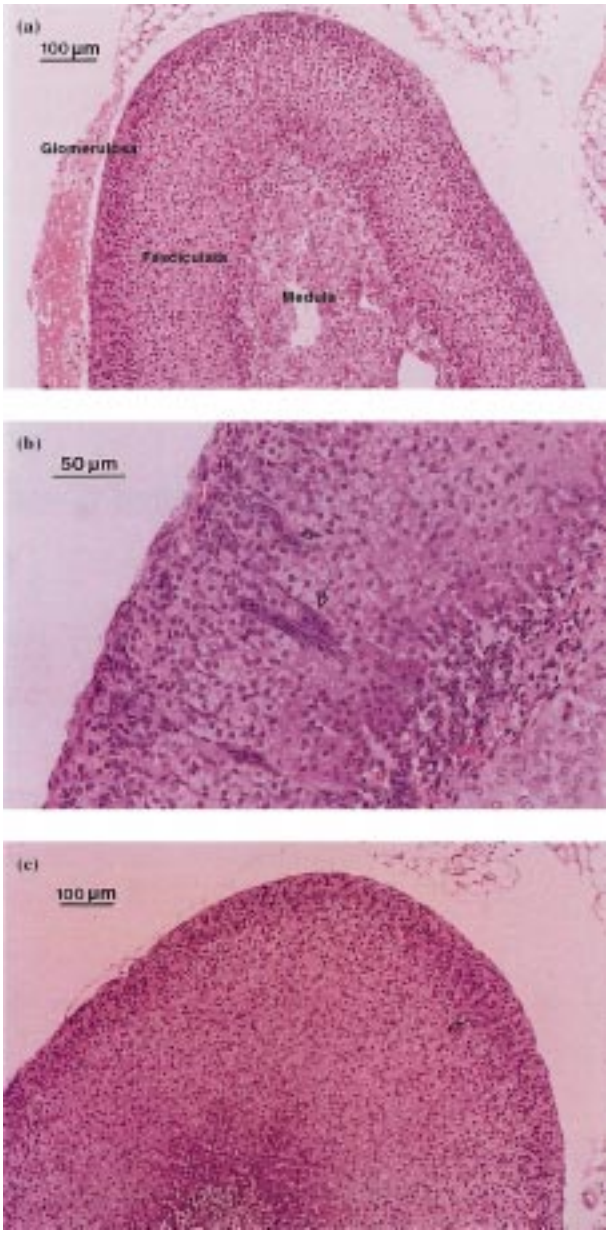

Figure 3. Microscopic appearance of the adrenal glands from athymic mice bearing HX118 human melanoma xenografts. Haematoxylin and eosin staining. (a) The adrenal with the residual capsular thickening and limited proliferation of cells in the zona glomerulosa as found in control mice bearing non-irradiated cutaneous melanoma. Magnification ×200. The adrenal with an extensive proliferation of cells in the zona glomerulosa (b) and activated zona fasciculata (c) as observed in 100% and 86%, respectively, of the investigated glands from mice intravenously injected with 0.17 MBq ^{131}I-MTB which delivered a radiation dose of 6 cGy to primary tumours. Magnification ×400 and ×200, respectively.

DISCUSSION

Melanoma is known for its exceptional malignancy [3]. However, the subline of HX118 human melanoma xenograft used for the study had almost lost its ability to spontaneously metastasise. The tumour, therefore, was a suitable model to investigate whether very low-doses of ionising radiation deposited in the lesion can restore the metastatic ability of its cells. Indeed, the lowest investigated dose of 6 cGy, that is well below therapeutic values, initiated the growth of metastases in all animals. The frequency of appearance of metastases, their size, as well as the distance from the cutaneous tumours from which they originated suggest that a significant number of cells was released from the primary lesions and the process was initiated immediately after deposition of the radioisotope in melanoma cells. Both the size of metastases and their distance from the primary tumour were reduced with the gradually increased radioactivity of ^{131}I stably localised in melanoma cells. Obviously, it was necessary for the radioisotope's radioactivity to decay to the level essential to initiate the metastatic cascade. However, since the observation time was the same for the majority of animals, such a delay in the dissemination of cells from the primary lesions deferred the growth of distant metastases, limiting the detectable spread to satellite lesions and/or to cell invasion of the vasculature surrounding the primary tumour. Incomplete encapsulation of irradiated tumours found regardless of the radioactivity of ^{131}I employed and accompanied by the invasion of the normal tissue surrounding the lesions further supports the observation. It is very unlikely that the progressive increase in necrosis in irradiated tumours contributed to the pattern of dissemination. Since melanoma spreads through the host vasculature localised predominantly in the normal tissues surrounding the lesions and less often deep within the tumour mass, metastases derive primarily from cells growing at the tumour margin, that is those that remain viable even in the highly necrotic lesions.

Histopathological investigation revealed that inflammatory infiltration of cutaneous tumours, as well as immune responses observed frequently at the residual level in non-irradiated melanoma lesions, significantly changed after exposure to ionising radiation. Since neither metastases nor immune reactions similar to those observed in mice injected with ^{131}I-MTB were found in animals given cold MTB, it seems that ionising radiation at the applied doses initiated both the metastatic process and inflammatory reactions, indicating that there may be a causative link between the two phenomena.

It was conspicuous that the quality and quantity of the immune responses associated with metastasising tumours were dependent on the radiation dose deposited in the cutaneous melanoma. Whilst the radioactivity of 0.17, 1.4 and 1.7 MBq stimulated inflammatory reactions, with the maximum efficacy achieved with 1.4 MBq ^{131}I-MTB, 0.6 MBq inhibited almost entirely both local (tumour) and humoral immune responses. The induced immune reactions affected the growth of primary (cutaneous) melanoma also in a dose-dependent manner, that is particularly in animals injected with 1.4 MBq of ^{131}I. Consequently, the overall reduction of the tumour growth by administration of 1.4 MBq ^{131}I was more significant than that by higher radioactivity which, being more potent in damaging melanoma cells directly, but less so in stimulating immune responses, did not diminish the growth of the lesions in an adequate manner. There was also a strong correlation between the radiation dose-dependent

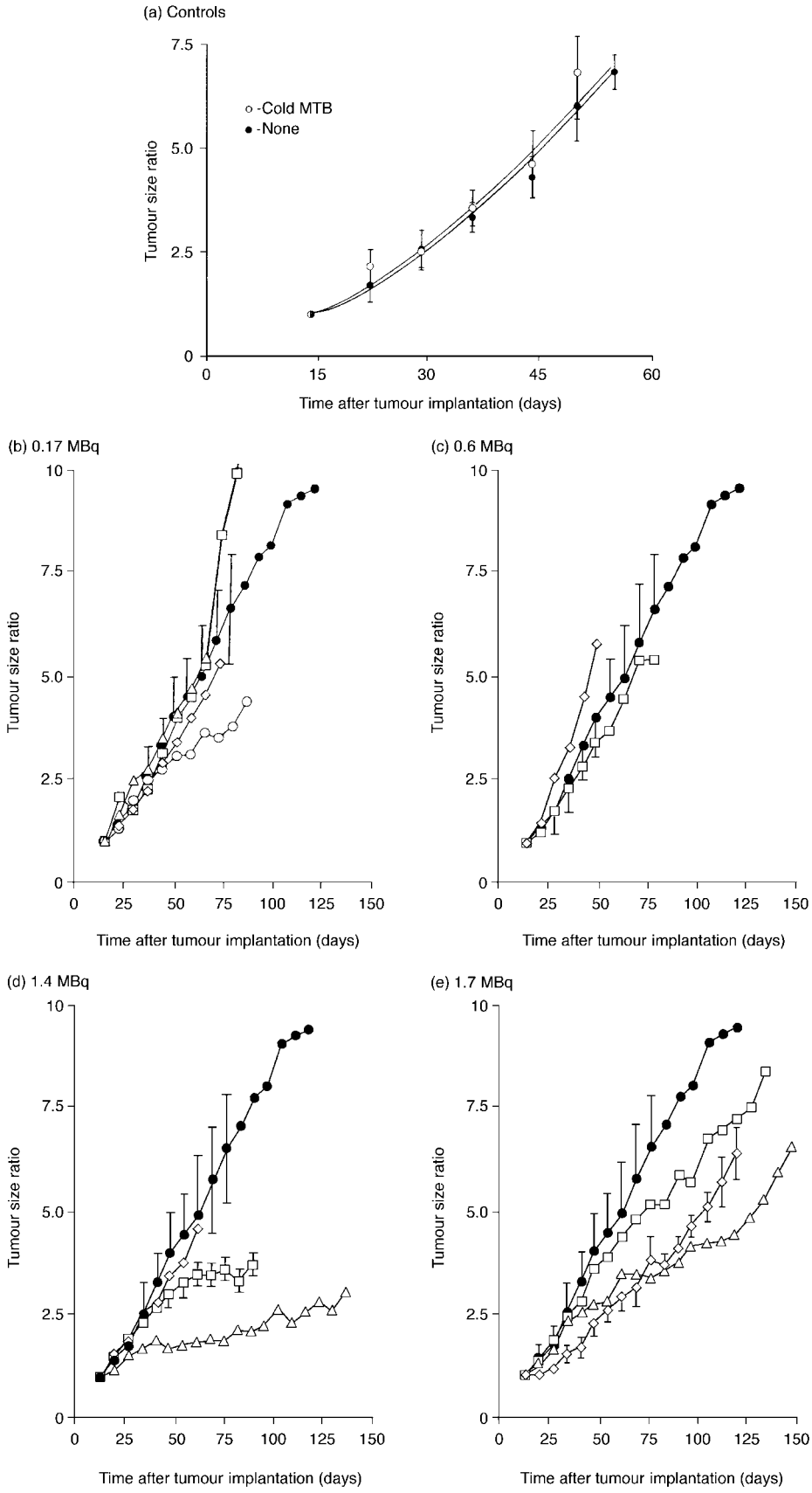

Figure 4. Growth rate of pigmented HX118 human melanoma xenografts inoculated subcutaneously into athymic mice from which metastases subsequently derived. (a) Control (cold MTB and none); (b) 0.17 MBq of ^{131}I-MTB (○,◇,□); (c) 0.6 MBq of ^{131}I-MTB (◇,□); (d) 1.4 MBq of ^{131}I-MTB (△,◇,□); (e) 1.7 MBq of ^{131}I-MTB (△,◇,□). Error bars: ± S.D. (standard deviation) ● Control (cold MTB and none).

degree of inflammatory infiltration of the primary melanoma, the tumour pigmentation and the activation of the adrenal cortex, suggesting that the immune system–adrenal axis feedback loop had been triggered [22]. Moreover, the extent of radiation-stimulated immune responses correlated with the acceleration of the conversion of cells from zona glomerulosa to those of the zona fasciculata—the phenomenon associated with the increased release of glucocorticoids by the adrenals. (The adrenal cortex regulates immune and inflammatory reactions by the release of glucocorticoids—the molecules that interrupt the enzymatic pathways involved in the synthesis of the mediators of the immune processes, namely, eicosanoids. In turn, the release of glucocorticoids from the adrenal cortex is stimulated by adrenocorticotropic hormone (ACTH) secreted from the adenohypophysis upon activation of the pituitary–adrenal counter-regulation of inflammation, as well as by ACTH and melanocyte stimulating hormane (α-MSH) released by melanoma cells [23] and lymphocytes themselves [24]. The activation of the latter small loop is supported by the extensive pigmentation of melanoma cells localised in the immediate vicinity of lymphocytes and inflammatory cells surrounding and infiltrating cutaneous tumours (ACTH and α-MSH also stimulate pigmentation of melanoma cells [25])).

Taken together, the concomitant occurrence of metastasis with the induced immune reactions, increased pigmentation of the primary melanomas and suprarenal activation responsible for regulating the immune responses by the release of glucocorticoids implicates eicosanoids as molecules initiating the observed processes and ionising radiation as responsible for their enhanced synthesis. Indeed, recent studies indicate that ionising radiation alters production of eicosanoids reviewed in [26] and that some eicosanoids initiate the metastatic cascade [27–29]. However, it remains unclear how the synthesis of eicosanoids is triggered by radiation since emitted electrons and photons cannot activate directly the relevant enzymes. It appears that hydrogen peroxide generated by ionising radiation via water radiolysis (each cell consists of approximately 80% of water) [30, 31] is responsible for initiating the reactions. This molecule has been shown to be immediately involved in stimulating relevant enzymatic pathways [32, 33] and H_2O_2-mediated cellular responses are eicosanoid-dependent [34, 35]. Both ionising radiation and H_2O_2 affect eicosanoid production in a biphasic manner (that is the pattern governing also ^{131}I-induced metastasis). Acceleration is observed within a low-dose range, whereas further elevation of radiation doses or H_2O_2 concentration results in a rapid termination of eicosanoid synthesis [36, 37]. Thus, if the hypothesis is correct, H_2O_2 formed in melanoma cells at the appropriate concentrations due to water radiolysis caused by radiation, should stimulate production of the relevant eicosanoids and favour tumour spread, whereas a further increase of radiation doses should gradually terminate the reaction. Indeed, the lowest ^{131}I radioactivity used proved to be the most effective in inducing metastases, but higher radioactivities gradually diminished the efficacy of the process. We have, therefore, calculated whether 6 cGy—the radiation dose most effective in initiating metastasis—generated H_2O_2 at concentrations suitable for stimulating synthesis of the eicosanoids.

Our previous experiments revealed that 10^{-13} moles of H_2O_2 per cell when added extracellularly was needed to obtain maximum stimulation of eicosanoid synthesis (10^{-12} moles per cell was already borderline in inhibiting the enzymes in question) [34]. This compares with 6×10^{-15} moles and 2×10^{-14} moles of H_2O_2 per cell, respectively, when H_2O_2 is produced by low linear energy transfer (LET) radiation simultaneously within and outside irradiated cells [38]. Since G value for H_2O_2 formed in water by β-electrons emitted by ^{131}I amounts to approximately 0.65 (the G value is defined as the number of molecules changed per 100 eV transferred to the system [30]), 1 Gy of radiation dose from the radioisotope produces 6.7×10^{-8} M H_2O_2 ($=0.65\times 1.036\times10^{-7}$ M H_2O_2) and, therefore, 6 cGy 4.04×10^{-9} M H_2O_2. This is equivalent to 5.3×10^{-15} moles H_2O_2 per cell since 1 g of the tumour consists of approximately 7.6×10^{8} melanoma cells [11].

The calculations reveal, therefore, that the radiation dose of 6 cGy delivered by ^{131}I generates hydrogen peroxide at the concentrations determined as optimal for stimulating synthesis of eicosanoids. This makes the proposed mechanism underlying radiation-induced metastasis and accompanying immune responses even more attractive.

In summary, data presented in this paper demonstrate that very low and well defined doses of ionising radiation are effective in inducing metastatic spread from primary melanomas. Reciprocity of the process versus radiation doses at the range investigated suggests the involvement of enzyme-mediated reactions induced by the radiation. Concomitant activation of the immune system–adrenal axis loop controlling synthesis of eicosanoids indicates that the enzymatic reactions triggered by the radiation are responsible for the production of these molecules.

1. Delaunay MM. Prognostic factors in melanoma. In Kirkham N, Cotton DWK, Lallemand RC, White J, Rosin RD, eds. *Diagnosis and Management of Melanoma in Clinical Practice*. Berlin, Springer, 1992, 97–112.
2. Epstein JH. Photocarcinogenesis, skin cancer and aging. *J Am Acad Dermatol* 1983, **9**, 487–502.
3. MacKie RM. Malignant melanoma. In *Skin Cancer*. London, Martin Dunitz Ltd, 1989, 178–201.
4. Shea CR, Parrish JA. Non-ionizing radiation and the skin. In Goldsmith LA, ed. *Physiology, Biochemistry and Molecular Biology of the Skin*. Oxford, Oxford University Press, 1991, 910–927.
5. Bowden GT, Joseloff E, Gupta A. The rôle of radiation in initiation, promotion and progression of carcinogenesis. In Hagen U, Harder D, Jung H, Streffer C, eds. Radiation Research 1895–1995. Würzburg, Universitätsdruckerei H. Stürtz AG, 1995, 584–587.
6. Boecker BB, Griffith WC. Cancer from internal emitters. In Hagen U, Harder D, Jung H, Streffer C, eds. Radiation Research 1895—1995. Würzburg, Universitätsdruckerei H. Stürtz AG, 1995, 1122–1125.
7. Epidemiological Studies of Radiation Carcinogenesis. In Sources and Effects of Ionizing Radiation, Annex A, UNSCEAR Report to the General Assembly. New York, United Nations, 1994.
8. von Essen CF. Radiation enhancement of metastasis: a review. *Clin Expl Metastas* 1991, **9**, 77–104.
9. Link EM. Targeted radiotherapy for disseminated melanoma. *Tumor Targeting* 1995, **1**, 309–316.
10. Link EM, Lukiewicz S. A new radioactive drug selectively accumulating in melanoma cells. *Eur J Nucl Med* 1982, **7**, 469–473.
11. Link EM, Brown I, Carpenter RN, Mitchell JS. Uptake and therapeutic effectiveness of ^{125}I- and ^{211}At-methylene blue for pigmented melanoma in an animal model system. *Cancer Res* 1989, **49**, 4332–4337.
12. Link EM, Costa DC, Lui D, Ell PJ, Blower PJ, Spittle MF. Targeting disseminated melanoma with radiolabelled methylene blue: comparative bio-distribution studies in man and animals. *Acta Oncol* 1996, **35**, 331–341.
13. Link EM, Blower PJ, Costa DC, *et al*. Early detection of melanoma metastases with radioiodinated methylene blue. *Eur J Nucl Med* 1998, **25**, 1322–1329.

14. Link EM. Targeting melanoma with $^{211}At/^{131}I$-methylene blue: pre-clinical and clinical experience. *Hybridoma* 1999, **18**, 77–82.
15. Link EM, Carpenter RN. ^{211}At-methylene blue for targeted radiotherapy of human melanoma xenografts: treatment of micrometastases. *Cancer Res* 1990, **50**, 2963–2967.
16. Link EM, Carpenter RN. ^{211}At-methylene blue for targeted radiotherapy of human melanoma xenografts: treatment of cutaneous tumors and lymph node metastases. *Cancer Res* 1992, **52**, 4385–4390.
17. Link EM, Michalowski AS, Rösch F. ^{211}At-methylene blue in targeted radiotherapy of disseminated melanoma. *Pigment Cell Res* 1994, **7**, 358–362.
18. Link EM, Carpenter RN, Hansen G. ^{211}At-methylene blue for targeted radiotherapy of human melanoma xenografts: dose fractionation in the treatment of cutaneous tumours. *Eur J Cancer* 1996, **32A**, 1240–1247.
19. Link EM, Michalowski AS, Rösch F. ^{211}At-methylene blue for targeted radiotherapy of disseminated melanoma: microscopic analysis of tumour versus normal tissue damage. *Eur J Cancer* 1996, **32A**, 1986–1994.
20. Baker FJ, Silverton RE, eds. *Introduction to Medical Laboratory Technology*. London, Butterworth Scientific, 1976 299–426.
21. Armitage P. *Statistical Methods in Medical Research*. Oxford, Blackwell, 1980.
22. Stenberg EM, Chrousos GP, Wilder RL, Gold PW. The stress response and the regulation of inflammatory disease. *Ann Intern Med* 1992, **117**, 857–866.
23. Herlyn M, Shih I-M. Interactions of melanocytes and melanoma cells with the microenvironment. *Pigment Cell Res* 1994, **7**, 81–88.
24. Cooke A. Regulation of the immune responses. In Roitt I, Brostoff J, Male D, eds. *Immunology*, 3rd ed. London, Mosby, 1993, 9.1–9.14.
25. Eberle AN, de Graan PNE, Baumann JB, Girard J, van Hees G, van de Veerdonk CG. Structural requirement of α-MSH for the stimulation of MSH receptors on different pigment cells. In Bagnara J, Klaus SN, Paul E, Schartl M, eds. *Biological, Molecular and Clinical Aspects of Pigmentation*. Tokyo, Tokyo University Press, 1985, 191–196.
26. Milas L, Hanson WR. Eicosanoids and radiation. *Eur J Cancer* 1995, **31A**, 1580–1585.
27. Marnett LJ, Honn KV. Overview on eicosanoids and cancer. *Cancer Metastas Rev* 1994, **13**, 237–239.
28. Tang DG, Honn KV. Lipoxygenase metabolites and cancer metastasis. In AICR, ed. *Dietary Fats and Cancer*. New York, Plenum Press, 1997.
29. Onoda JM, Kantak SS, Piechocki MP, *et al*. Inhibition of radiation-enhanced expression of integrin and metastatic potential in B16 melanoma cells by a lipoxygenase inhibitor. *Radiation Res* 1994, **140**, 410–418.
30. Alpen EL. *Radiation Biophysics*. New Jersey, Prentice Hall, 1990, 91–97.
31. Peak MJ, Pilas B, Dudek EJ, Peak MJ. DNA breaks caused by monochromatic 365 nm radiation or hydrogen peroxide and their repair in human epithelioid and xenoderma pigmentosum cells. *Photochem Photobiol* 1991, **54**, 197–203.
32. Lands WEM. Enzymic assays of hydroperoxides. In Rice-Evans C, Halliwell B, eds. *Free Radicals, Methodology and Concepts*. London, Richelieu Press, 1988, 213–224.
33. Schilstra MJ, Veldink GA, Vliegenthart JFG. Kinetic analysis of the induction period in lipoxygenase catalysis. *Biochemistry* 1993, **32**, 7686–7691.
34. Link EM. Enzymic pathways involved in cell response to H_2O_2. *Free Radical Res Comm* 1990, **11**, 89–97.
35. Link EM. Inflammation and a mechanism of hydrogen peroxide cytotoxicity. In Poli G, Albano E, Dianzani MU, eds. *Free Radicals: From Basic Science to Medicine*. Basel/Switzerland, Birkhäuser.
36. Deby C, Deby-Dupoint G. Oxygen species in prostaglandin biosynthesis *in vitro* and *in vivo*. In Bannister WH, Bannister JV, eds. *Biological and Clinical Aspects of Superoxide and Superoxide Dismutase*. Amsterdam, Elsevier, 1980, 84–97.
37. Ziboh VA, Mallia E, Morhart E, Taylor JR. Induced biosynthesis of cutaneous prostaglandins by ionizing radiation. *Proc Soc Exp Biol Med* 1982, **169**, 386–391.
38. Link EM. In Wardman P, Willson RL, eds. *Free Radical Biochemistry and Radiation Injury*. *Br J Cancer* 1987, **55**(Suppl. VIII), 110–112.

Acknowledgements—We are very grateful to Professor G. Vinson of the Department of Biochemistry, St. Bartholomew's and the Royal London School of Medicine and Dentistry, for the stimulating discussion concerning the mechanism of ACTH and α-MSH mediated activation of the adrenal cortex.