

The prevalence of interferon-alpha transcription defects in malignant melanoma

Karen L. Price^a, Meenhard Herlyn^b, Carolyn L. Dent^c, Dirk R. Gewert^d and Claire Linge^a

The type I interferons, interferon-alpha (IFN- α) and interferon-beta (IFN- β), are situated on the short arm of chromosome 9, specifically 9p21-22. This locus lies very close to an area that is deleted or rearranged in nearly half of all melanomas tested. The identification of 9p rearrangements in both melanoma precursor lesions (dysplastic naevi) and primary lesions has implicated the 9p locus in the early stages of melanoma development. Recent evidence has demonstrated that metastatic melanoma cell lines have a specific loss of IFN- α gene expression, a defect that appears to occur at the level of transcription. In this study, we examined the expression of IFN- α in cell lines isolated from the various stages of melanoma progression, with a view to determine the prevalence of the IFN- α transcription defects exhibited by malignant melanoma, and to assess whether the loss of IFN- α expression was particular to a certain stage of melanoma progression. We showed that all the melanoma cell lines tested ($n=20$) demonstrated an inability to express IFN- α , a defect that was reflected in the apparent inactivity of the IFN- α promoter. These defects were found to occur in cells

isolated from early melanomas, lending support to the hypothesis that IFN- α has a role in the aetiology of malignant melanoma. *Melanoma Res* 15:91-98 © 2005 Lippincott Williams & Wilkins.

Melanoma Research 2005, 15:91-98

Keywords: Interferon-alpha, melanocytes, melanoma, melanoma progression

^aRAFT Institute of Plastic Surgery, Mount Vernon Hospital, Northwood, Middlesex HA6 2RN, UK, ^bThe Wistar Institute, Molecular and Cellular Oncogenesis Program, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, USA, ^cSangamo BioSciences, Inc., Richmond, California 94804, USA and ^dBioLauncher Ltd., Cambridge CB2 2LG, UK.

Sponsorship: This research was funded by RAFT, registered charity number 299811.

Correspondence and requests for reprints to Dr Claire Linge, RAFT Institute of Plastic Surgery, Mount Vernon Hospital, Northwood, Middlesex HA6 2RN, UK. Tel: (+44) 1923 844557; fax: (+44) 1923 844031; e-mail: lingec@raft.ac.uk

Received 14 July 2004 Accepted (after revision) 21 January 2005

Introduction

The type I interferons [interferon-alpha (IFN- α) and interferon-beta (IFN- β)] have been shown to exhibit strong antiviral, antiproliferative and immunomodulatory effects on a variety of cell types, including melanoma cell lines [1-3], and have since been proven to be highly effective antitumour agents. Indeed, one of the better therapies for the treatment of metastatic malignant melanoma is IFN- α , which shows reported response rates between 20 and 46%, with one-third of these responses being durable [4-6].

An indication of the importance of IFNs in malignant melanoma has come from reports that a 2-3 megabase region, 9p13-22, is deleted or rearranged in nearly half of all melanomas tested [7,8]. Rearrangements on the short arm of chromosome 9 have been reported in both melanoma precursor lesions (dysplastic naevi) and primary lesions [9]. This region of loss lies very close to, but does not include, the type I IFN locus, which is situated at 9p21-22 [10]. Taken together, these observations led to the proposal by Linge *et al.* [11] that this area contains a *cis*-acting dominant control domain that regulates the expression of the IFN genes, in a similar

manner to that observed for the regulation of the β -globin gene family [12].

Although their original hypothesis was not entirely correct, a novel finding was presented by Linge *et al.* [11]. They reported that normal human melanocytes produce IFN- β and IFN- α following viral induction, whereas their malignant counterparts, the melanoma cell lines, are only able to produce IFN- β under the same conditions. Linge *et al.* [11] further demonstrated that the translation mechanism of IFN- α and the secretion of the IFN- α protein were intact for all the melanoma cell lines tested, because, on transfection with a constitutive IFN- α_{2b} expression plasmid, the conditioned medium was found to contain high levels of IFN- α . The defect therefore appeared to occur at the level of transcription, a proposition supported by the finding that none of the melanoma cell lines tested showed significant activity of the IFN- α_1 promoter above background, whereas it was activated in the normal human melanocyte lines under the same conditions [11].

As a result of their findings, Linge *et al.* [11] proposed that the defect in IFN- α gene transcription may be a

common and therefore important event in the development and/or progression of malignant melanoma. However, only seven melanoma cell lines were investigated by Linge *et al.* [11], all of which were derived from metastatic disease. In this study, we further investigate these findings by examining IFN- α expression by melanoma cell lines derived from the different stages of melanoma progression (radial growth phase, vertical growth phase and metastases). This study was undertaken with a view to determine the prevalence of the IFN- α transcription defects exhibited by malignant melanoma, and also to examine whether the loss of IFN- α expression was particular to a certain stage of melanoma progression.

Materials and methods

Cell culture

The melanoma cell lines, A375M [13], A375P [13], HMB-2 [14], DX-3 [15], LT-5 [15], SK23 (originally obtained from Sloan-Kettering Institute, New York, USA [16]), SK28 (originally obtained from Sloan-Kettering Institute [17]) and MJM [18], were cultured in RPMI 1640 (Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS) (Invitrogen), 1% L-glutamine (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen). The melanoma lines, WM98-1, WM852, WM3211, 451lu, WM1158, WM983A, WM1366, WM39, WM1552C, WM278, WM239A and WM9 [19,20], were cultured in a 4:1 mixture of MCDB153 (Sigma, Poole, Dorset, UK) and L-15 (Sigma), supplemented with 2% FCS, 1% L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.5 mg/ml insulin (Invitrogen). Normal human melanocyte cell strains ($n=4$) were initiated from neonatal foreskins, as described previously [11]. Cells were cultured in MCDB153 medium supplemented with 5×10^{-2} mM CaCl₂, 10^{-9} M cholera toxin (Sigma), 5 μ g/ml insulin, 5 μ g/ml transferrin (Sigma), 0.2 μ g/ml hydrocortisone (Sigma), 6.1×10^{-4} % (v/v) ethanolamine (Sigma), 14 μ g/ml phosphoethanolamine (Sigma), 4% chelated FCS, 1% L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

IFN secretion assay

Cells (2×10^5 per 35 mm dish) were plated out into 2 ml of the relevant normal growth medium and allowed to adhere for 48 h. The cells were then either induced by the addition of Sendai virus or mock-induced by the addition of the equivalent volume of medium and incubated at 37°C in 5% CO₂ for 24 h. The culture supernatant was then harvested, centrifuged to remove cell debris and assayed for IFN- α or IFN- β production using enzyme-linked immunosorbent assay (ELISA). For IFN- α ELISA, 96-well plates were coated overnight at 4°C with 100 μ l per well of 2 μ g/ml bovine anti-human IFN- α (Wellcome Foundation Ltd., Beckenham, Kent, UK). The wells were washed five times with 0.1% Tween 20 in phosphate-buffered saline (PBS) and blocked with

3% bovine serum albumin (BSA) in PBS overnight at 4°C. After washing, 100 μ l of sample was added per well and incubated for 1 h at 37°C. IFN- α was detected with a 1:2000 dilution of anti-IFN- α /horseradish peroxidase (HRP)-conjugated polyclonal antibody (Wellcome Foundation Ltd.). Antibody binding was visualized with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma). The reaction was read at an absorbance of 450 nm. The minimum detectable concentration of IFN- α using this ELISA is 0.2 ng/ml. Quantification of IFN- β was performed using the specific immuno-enzymetric assay IFN- β -EASIA kit (Medgenix Diagnostics, Fleurus, Belgium) in accordance with the supplier's instructions. The minimum detectable concentration of IFN- β using this kit is 0.01 ng/ml. The experiments were performed on three separate occasions and an average value was taken.

Polymerase chain reaction (PCR) amplification

In order to determine the presence of intact IFN- α genes, genomic DNA was extracted from cells as follows: 10^6 cells were resuspended in 100 μ l of buffer A [75 mM NaCl (BDH, Poole, Dorset, UK) and 25 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 (BDH)], lysed by the addition of 100 μ l of buffer B (10 mM Tris, pH 8.0 (BDH), 10 mM EDTA, pH 8.0, 1% sodium dodecyl sulphate (BDH) and 400 μ g/ml proteinase K) and incubated at 50°C for 3 h. The preparation was gently extracted with phenol (Invitrogen), phenol-chloroform (1:1) and chloroform (BDH), followed by ethanol precipitation. The presence of an intact IFN- α gene was verified in melanoma cell lines and normal melanocyte cell lines by PCR, as described previously [21]. The primers used were: BK40, CAC GAA GCT TAT AAC CAC CAC CAT GGC CTT GAC CTT TGC TTT ACT GG (which includes the first 25 bases of the translated sequence of IFN- α_2); BK41, GTT AGA ATT CTA GAT CAT TCC TTA CTT ACT TCT TAA ACT TTC TTG C (which includes sequence complementary to 28 bp of the 3' translated sequence prior to and including the termination codon of IFN- α_2). PCR amplifications were performed using the Hybaid thermal cycler (Hybaid, Ashford, Middlesex, UK) with the following programme: 30 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 2 min, with a final extension step of 72°C for 7 min. The result shown is a representative agarose gel for at least three independent experiments.

Subcloning and sequencing

To determine the expression of active IFN- α subtypes in normal human melanocyte cells, mRNA was prepared from 1×10^6 cells following 6 h of stimulation with Sendai virus, according to the instruction manual (Promega, Southampton, Hampshire, UK). Reverse transcriptase-PCR was performed using the primers described above and the PCR product was subcloned into pUC18 (Invitrogen). The bacterial strains were harvested for

small-scale preparations according to the Qiagen kit instructions (Qiagen, Crawley, West Sussex, UK). The DNA sequence was determined using Sequenase version 2.0 T7 DNA polymerase kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Transfection and secreted alkaline phosphatase assay

Cells (6×10^4) were plated out into 48-well plates, 24 h prior to transfection. The wells were then washed three times with serum-free medium. Serum-free medium (200 μ l) containing 2 μ g/ml Transfectam (Promega) and 16 μ g/ml of the appropriate plasmid DNA were added to each well and the plates were incubated for 1 h at 37°C. The constructs used to analyse the activity of the type I IFN promoters contained the virus response element (VRE, -110 to -51) of the relevant IFN gene in front of the thymidine kinase minimal promoter, which drives the expression of the reporter secreted alkaline phosphatase (SAP) [22]. After this time, the medium was replaced with 200 μ l of normal growth medium, with or without Sendai virus, and incubated for 24–48 h, when the supernatants were harvested for assay of the reporter gene. Medium samples were incubated at 65°C for 20 min, allowed to cool and 50 μ l of sample (or sample dilutions) was incubated with 150 μ l of substrate solution (2 mg/ml *p*-nitrophenyl phosphate in 1 M diethanolamine and 0.5 mM MgCl₂ at pH 9.5) in 96-well plates. The plates were left at room temperature for 2–24 h until the reaction had developed, and were read at 405 nm. The raw data from the IFN constructs were normalized for each cell line by calculating the value as a percentage of the activity of the positive control plasmid (pILSAP), which contains the human β -actin promoter driving the reporter gene [11], to take into account any differences in transfection efficiency and general transcription activity.

The experiments were performed on three separate occasions and an average value was taken.

Results

Expression of IFN- α in melanoma

The melanoma cell lines ($n = 20$) and normal human melanocyte lines ($n = 4$) were examined for their ability to secrete IFN- α and IFN- β in response to Sendai virus. The majority of the melanoma cell lines were able to produce endogenous IFN- β following virus induction (> 8.2 ng per 10^6 cells), with the exception of SK28, WM9 and WM983A (Table 1). In contrast, none of the melanoma cell lines tested produced detectable levels of IFN- α under the same conditions. However, normal human melanocyte lines were found to secrete both IFN- α and IFN- β in response to Sendai virus (34.2 and 9.3 ng per 10^6 cells, respectively). The melanoma lines were derived from the various stages of melanoma progression (Table 1), suggesting that the loss of IFN- α production could be a widespread occurrence in malignant melanoma and could occur early in the progression of malignant melanoma.

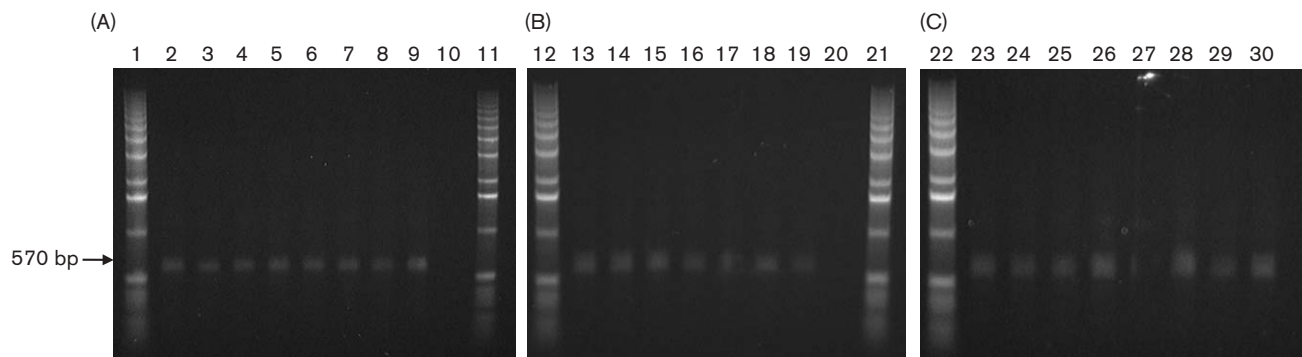
Demonstration of the presence of IFN- α genes

Having demonstrated that none of the melanoma cell lines tested produced detectable levels of endogenous IFN- α in response to Sendai virus, we went on to establish whether each of the melanoma cell lines contained intact IFN- α genes. Genomic DNA was prepared from each melanoma cell line and used as a template for PCR amplification, with the use of specific primers, BK40 and BK41, which amplify the IFN- α_1 , IFN- α_2 , IFN- α_5 and IFN- α_{13} genes. The results of the PCR (Fig. 1) showed that a band of the correct size of 570 bp was obtained for all of the cell lines tested

Table 1 Levels of the type I interferons, IFN- α and IFN- β , produced in Sendai virus-induced melanoma cell lines ($n = 20$). Normal human melanocytes ($n = 4$) were used as a positive control. The origin of the melanoma cell lines is also shown. Results shown are the average value of three independent experiments

Melanoma cell line	IFN- α (ng/ 10^6 cells)	IFN- β (ng/ 10^6 cells)	Stage of melanoma progression
WM1552C	0	>40	Radial growth phase
WM3211	0	>40	Radial growth phase
WM39	0	>40	Vertical growth phase
WM983A	0	0	Vertical growth phase
WM98-1	0	>40	Vertical growth phase
WM1366	0	>40	Vertical growth phase
WM278	0	17.6	Vertical growth phase
A375M	0	>40	Metastasis
A375P	0	>40	Metastasis
SK28	0	0	Metastasis
SK23	0	21.2	Metastasis
HMB-2	0	12.3	Metastasis
LT-5	0	>40	Metastasis
DX-3	0	>40	Metastasis
WM852	0	>40	Metastasis
451lu	0	14.2	Metastasis
WM9	0	0	Metastasis
WM239A	0	>40	Metastasis
WM1158	0	8.2	Metastasis
MJM	0	>40	Metastasis
Normal human melanocytes	34.2	9.3	

Fig. 1



One per cent agarose gel showing the results of polymerase chain reaction (PCR) amplification of interferon-alpha (IFN- α) genes from melanoma cell lines ($n=20$) and a representative normal human melanocyte line (NM; $n=4$). Results shown are representative of at least three independent experiments. (A) Lane 1, 1 kb DNA ladder. Lane 2, NM. Lane 3, A375M. Lane 4, A375P. Lane 5, DX-3. Lane 6, LT-5. Lane 7, SK28. Lane 8, SK23. Lane 9, HMB-2. Lane 10, negative control. Lane 11, 1 kb DNA ladder. (B) Lane 12, 1 kb DNA ladder. Lane 13, MJM. Lane 14, WM852. Lane 15, WM1366. Lane 16, 451lu. Lane 17, WM9. Lane 18, WM983A. Lane 19, WM278. Lane 20, negative control. Lane 21, 1 kb DNA ladder. (C) Lane 22, 1 kb DNA ladder. Lane 23, WM239A. Lane 24, WM39. Lane 25, WM98-1. Lane 26, WM1158. Lane 27, no sample. Lane 28, WM3211. Lane 29, WM1552C. Lane 30, NM.

($n=20$), indicating that at least one of the above listed IFN- α genes was present in these cell lines. Genomic DNA preparations from normal human melanocyte lines ($n=4$) were used as positive controls. A negative control consisting of the PCR mixture excluding template genomic DNA was included to ensure that any positive results were not simply due to contamination of the PCR mixture.

Identification of active IFN- α genes in virus-stimulated normal human melanocytes

In order to identify the various IFN- α subtypes active in normal human melanocyte cells following Sendai virus induction, IFN- α -specific primers, BK40 and BK41, were used to amplify coding sequences from total cellular RNA isolated from normal human melanocyte lines after Sendai virus infection. This fragment was then subcloned into the plasmid pUC18 and sequenced. Half (5/10) of the clones corresponded to IFNA1 or IFNA13, one was an IFNA5 allele and four were IFNA2.

Determination of the activity of the type I IFN genes in melanoma

As the IFN- α_2 gene has been demonstrated to be expressed by Sendai virus-induced normal human melanocyte lines, we went on to determine whether the activity of this specific IFN- α promoter could be demonstrated following viral induction in normal human melanocyte lines ($n=4$) and melanoma cell lines ($n=20$). As a control for Sendai virus activation, the activity of the IFN- β promoter was also examined. The IFN- β promoter construct was activated by viral induction in all the melanoma cell lines tested, with the exception of WM9 and WM983A (Fig. 2). Interestingly,

the SK28 cell line, which did not secrete any detectable levels of endogenous IFN- β in response to Sendai virus ([11] and Table 1), demonstrated an active IFN- β promoter. In contrast, the IFN- α_2 promoter was not activated by virus in any of the melanoma cell lines tested (Fig. 3), whereas, in the normal human melanocyte lines, the IFN- α_2 promoter construct was significantly induced above background following viral induction (Fig. 3).

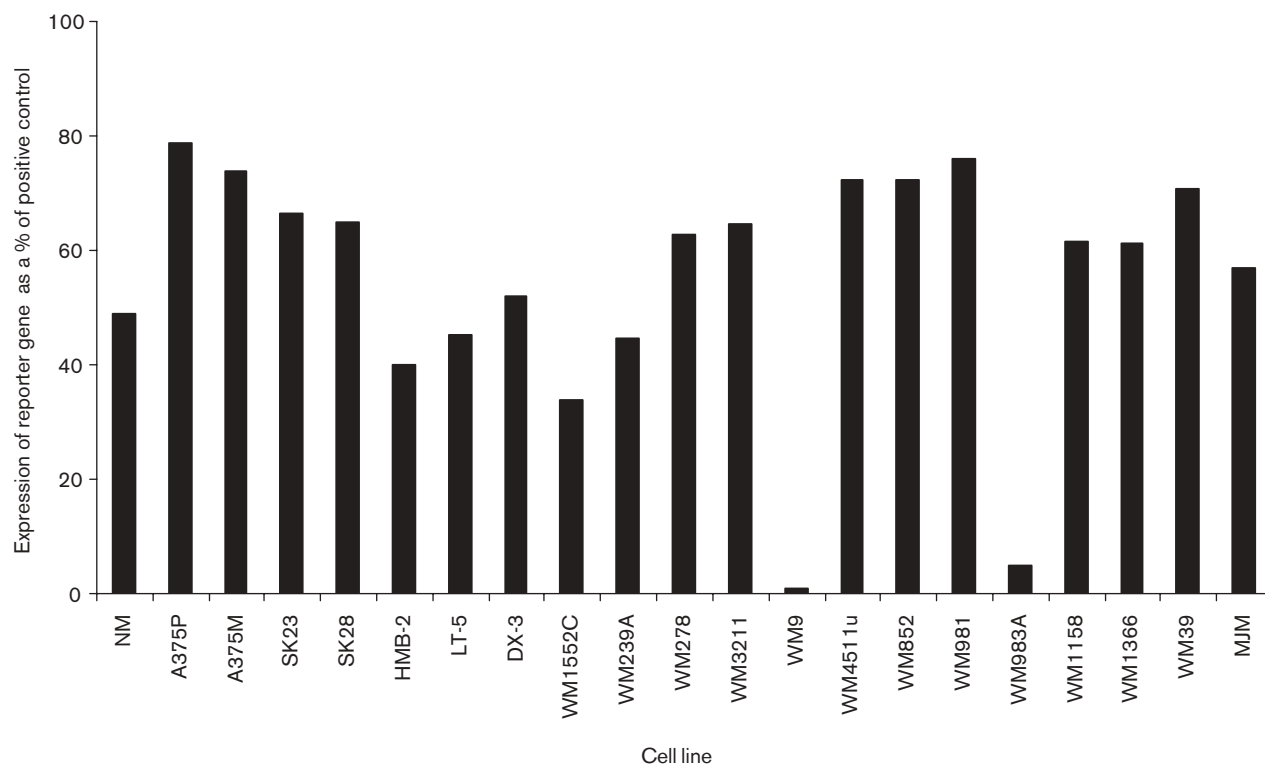
Investigation of the activity of the promoters of immediate-early and late response IFN- α genes

Murine IFN- α genes can be divided into two groups: an immediate-early response gene (IFN- α_4) and a set of genes that display delayed induction, consisting of at least IFN- α_2 , IFN- α_5 , IFN- α_6 and IFN- α_8 [23]. As the activity of IFN- α_2 , a putative delayed response human IFN- α gene, was found to be absent in melanoma cell lines, it was important to investigate whether the promoter region of a potential immediate-early response human IFN, IFN- α_4 , was active in the melanoma cell lines and normal human melanocyte lines. We demonstrated that the IFN- α_4 promoter was not active following viral induction in any of the melanoma cell lines tested. Again, the IFN- α_4 promoter was found to be activated in the normal human melanocyte lines following viral induction (Fig. 4).

Discussion

The results of this study show that normal human melanocyte lines produce both IFN- β and IFN- α in response to virus (9.3 and 34.2 ng per 10^6 cells, respectively) (Table 1), as described previously [11]. Furthermore, the majority of melanoma cell lines ($n=20$) secrete significant levels of IFN- β (>8.2 ng per 10^6

Fig. 2



Graph showing the activity of the interferon-beta (IFN- β) promoter in melanoma cell lines ($n=20$) and normal human melanocyte cells (NM; $n=4$). Results shown are the average value of three independent experiments.

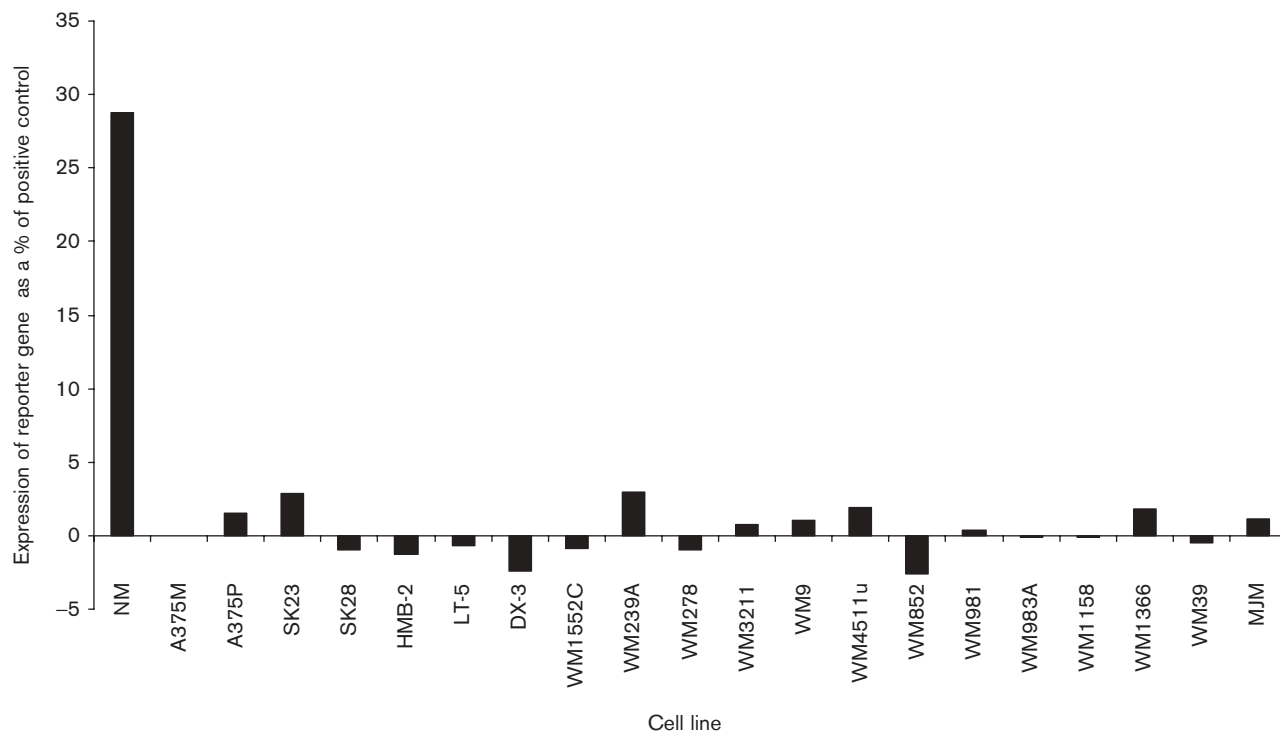
cells), with the exception of WM983A (vertical growth phase), SK28 (metastatic) and WM9 (metastatic), following viral induction (Table 1). However, none of the melanoma cell lines tested produce detectable levels of IFN- α under similar conditions (Table 1). These results appear to suggest that the loss of IFN- α production could be a common event in malignant melanoma, and may even occur in cell lines derived from the early stages of melanoma progression (WM1552C and WM3211; Table 1). These results may indicate a role for IFN- α in the aetiology of malignant melanoma. Reports of rearrangements on the short arm of chromosome 9, close to the type I IFN locus, in both melanoma precursor lesions (dysplastic naevi) and primary lesions [9], lend further support to this hypothesis. To investigate whether the loss of IFN- α production has a role in the initiation/promotion of melanoma progression, it would be of interest to examine the expression of the type I IFN in melanoma precursors (common acquired melanocytic naevi and dysplastic naevi). However, it is possible that this phenomenon occurs simply as a consequence of the cells adapting to the culture conditions. To address this possibility, it would be important to examine IFN- β and IFN- α production in freshly isolated melanoma cells.

This defect in IFN- α production is not due to the deletion of all IFN- α genes, because, as shown by PCR amplification, all of the melanoma cell lines tested and normal human melanocyte lines contained at least one intact IFN- α gene (Fig. 1). Nevertheless, this result does not rule out mutational inactivation of these genes, although this would be unlikely given the number of active genes in this family.

Having demonstrated that all the melanoma cell lines contained at least one intact IFN- α gene, we wanted to examine the activity of an IFN- α promoter in these cells. In their original study, Linge *et al.* [11] demonstrated that the IFN- α_1 VRE region was active in virally stimulated normal human melanocytes. However, it is unclear whether normal human melanocytes are capable of expressing IFN- α_1 , IFN- α_{13} or both, due to the fact that IFN- α_1 and IFN- α_{13} are identical over the coding region of the gene [24]. To address this problem, we chose to determine the promoter activity of another IFN- α gene for further study, preferably one that has been demonstrated as active in normal human melanocytes.

Having demonstrated that, in response to viral induction, the IFN- α_2 gene was expressed in normal human

Fig. 3



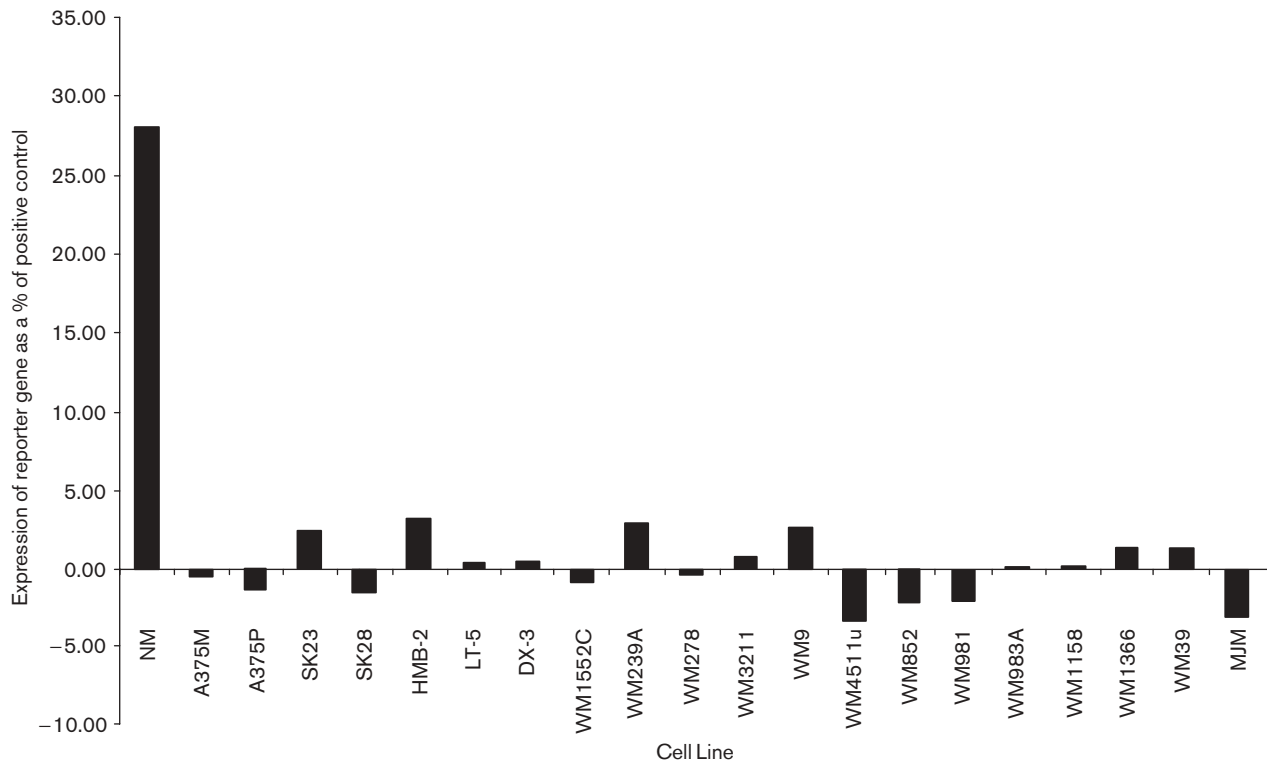
Graph showing the activity of the interferon- α_2 (IFN- α_2) promoter in melanoma cell lines ($n=20$) and normal human melanocyte cells (NM; $n=4$). Results shown are the average value of three independent experiments.

melanocytes; we next examined the activity of this specific IFN- α promoter in both normal human melanocytes and melanoma cell lines following viral induction. The results of the transfection experiments demonstrated that significant activation of the IFN- β promoter in response to viral infection occurred in the normal human melanocyte lines ($n=4$) and in the majority of the melanoma cell lines tested ($n=20$), with the exception of WM9 and WM983A (Fig. 2). The reason for the loss of IFN- β promoter activity in these cell lines was not addressed further in this study, but it would be of interest to compare the expression of the regulatory proteins in these lines with those that have strong IFN- β promoter activity (e.g. A375M) in the DNA mobility shift assay. This experiment might help to elucidate which transcription factors are essential for IFN- β promoter activity in the various melanoma cell lines. In contrast, the IFN- α_2 promoter was not active following viral infection in any of the melanoma cell lines tested (Fig. 3). However, the IFN- α_2 promoter was active in normal human melanocytes following induction with Sendai virus (Fig. 3).

Recently, Marie *et al.* [23] have reported that the murine IFN- α genes can be divided into two groups: an immediate-early response gene (IFN- α_4) and a set of genes that display delayed induction, consisting of at

least IFN- α_2 , IFN- α_5 , IFN- α_6 and IFN- α_8 . It was shown that the IFN- α_4 gene could be rapidly and directly induced in response to virus infection, without the need for ongoing protein synthesis. However, the delayed set of IFN- α genes was induced more slowly and was dependent on cellular protein synthesis. The authors demonstrated that one of the proteins that must be synthesized for the induction of the delayed gene set is IFN itself [23]. Their work suggested that the activation of the immediate-early IFN- α_4 and IFN- β promoters led to the production and secretion of the remaining type I IFNs. IFN- α_4 and/or IFN- β , secreted from virus-infected cells during the early response, would subsequently feedback on the cells through the type I IFN receptor, which would lead to the induction of the non-IFN- α_4 gene set. Whether the human IFN- α s can be so categorized has yet to be seen, but it remains possible that the inability of the melanoma cell lines to express IFN- α is simply due to a lack of feedback via IFN- α_4 or IFN- β . We therefore went on to examine the activity of the IFN- α_4 promoter in both melanoma and normal human melanocyte cells. Analogous to the transfection results with the IFN- α_2 promoter, activation of the IFN- α_4 promoter construct following viral induction was clearly seen only in normal human melanocytes but not in any of the 20 melanoma cell lines tested (Fig. 4).

Fig. 4



Graph showing the activity of the interferon-alpha₄ (IFN- α_4) promoter in melanoma cell lines ($n=20$) and normal human melanocyte cells (NM; $n=4$). Results shown are the average value of three independent experiments.

The results of these studies suggest that the loss of IFN- α production in cutaneous melanoma is a universal occurrence, and even occurs in melanoma cell lines derived from the early stages of melanoma progression. This loss of IFN- α expression is reflected in the inactivity of the IFN- α_2 and IFN- α_4 promoter regions, but not the IFN- β promoter, in the melanoma cell lines tested ($n=20$), suggesting that the defect lies within a transcription event common and specific to the IFN- α s rather than IFN- β . Furthermore, as the loss of IFN- α_2 and IFN- α_4 promoter activity is not only restricted to melanoma cell lines derived from metastatic disease, but seems to occur very early in melanoma progression, this finding lends support to the hypothesis that IFN- α has a role in the aetiology of malignant melanoma.

Acknowledgements

The authors are grateful to both Professor Roy Sanders and Professor Colin Green for their continuous encouragement and support.

References

- Isaac A, Lindenman J. Virus interference: the interferon. *Proc R Soc Lond* 1957; **147**:258–267.
- Pestka S, Langer JA, Zoon KC, Samuel CE. Interferons and their actions. *Annu Rev Biochem* 1987; **56**:727–777.
- Garbe C, Krasagakis K, Zouboulis CC, Schroder K, Kruger S, Stadler R, *et al.* Antitumor activities of interferon alpha, beta, and gamma and their combinations on human melanoma cells in vitro: changes of proliferation, melanin synthesis, and immunophenotype. *J Invest Dermatol* 1990; **95**(Suppl):S231–S237.
- Kirkwood JM, Strawderman MH, Ernstoff MS, Smith TJ, Borden EC, Blum RH. Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. *J Clin Oncol* 1996; **14**:7–17.
- Kirkwood JM, Ibrahim JG, Sondak VK, Richards J, Flaherty LE, Ernstoff MS, *et al.* High- and low-dose interferon alfa-2b in high-risk melanoma: first analysis of intergroup trial E1690/S9111/C9190. *J Clin Oncol* 2000; **18**(12):2444–2458.
- Kirkwood JM, Ibrahim JG, Sosman JA, Sondak VK, Agarwala SS, Ernstoff MS, *et al.* High-dose interferon alfa-2b significantly prolongs relapse-free and overall survival compared with the GM2-KLH/QS-21 vaccine in patients with resected stage IIB–III melanoma: results of intergroup trial E1694/S9512/C509801. *J Clin Oncol* 2001; **19**(9): 2370–2380.
- Cannon-Albright LA, Goldgar DE, Mey LJ, Lewis CM, Anderson DE, Fountain JW, *et al.* Assignment of a locus for familial melanoma, MLM, to chromosome 9p13-22. *Science* 1992; **258**:1148–1152.
- Fountain JW, Karayiorgou M, Ernstoff MS, Kirkwood JM, Vlock DR, Titus-Ernstoff L, *et al.* Homozygous deletions within human chromosome band 9p21 in melanoma. *Proc Natl Acad Sci USA* 1992; **89**:10557–10561.
- Fountain JW, Bale SJ, Housman DE, Dracopoli NC. Genetics of melanoma. *Cancer Surv* 1990; **9**:645–671.
- Diaz MO, Pomykala HM, Bohlander SK, Maltepe E, Malik K, Brownstein B, *et al.* Structure of the human type-I interferon gene cluster determined from a YAC clone contig. *Genomics* 1994; **22**:540–552.

- 11 Linge C, Gewert D, Rossmann C, Newton-Bishop JA, Crowe S. Interferon system defects in human malignant melanoma. *Cancer Res* 1995; **55**:4099-4104.
- 12 Curtain PT, Liu D, Liu W, Chang JC, Waikan Y. Human β -globin gene expression in transgenic mice is enhanced by a distant DNase I hypersensitive site. *Proc Natl Acad Sci USA* 1989; **86**:7082-7086.
- 13 Kozlowski JM, Hart IR, Fidler IJ, Hanna NA. A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. *J Natl Cancer Inst* 1984; **72**:913-917.
- 14 Siracky J, Blasko M, Borovancky J, Kovarik J, Svec J, Vrba M. Human melanoma cell lines: morphology, growth, and α -mannosidase characteristics. *Neoplasma* 1982; **29**:661-668.
- 15 Ormerod EJ, Everett CA, Hart IR. Enhanced experimental metastatic capacity of a human tumour line following treatment with 5-azacytidine. *Cancer Res* 1986; **46**:884-890.
- 16 Kozlowski JM, Fidler IJ, Campbell D, Xu Z, Kaighn ME, Hart IR. Metastatic behaviour of human tumour cell lines grown in the nude mouse. *Cancer Res* 1984; **44**:3522-3529.
- 17 Pukel CS, Lloyd KO, Travassos LR, Dippold WG, Oettgen HF, Old LJ. GD3, a prominent ganglioside of human melanoma. *J Exp Med* 1985; **155**: 1133-1147.
- 18 Kovarik J, Svejda J, Bucek J, Rejthar A, Dral B, Ninger E, et al. Establishment of cell line derived from human malignant melanoma. *Neoplasma* 1978; **25**(6):701-712.
- 19 Satyamoorthy K, DeJesus E, Linnenbach AJ, Krai B, Kornreich DL, Rendle S, et al. Melanoma cell lines from different stages of progression and their biological and molecular analysis. *Melanoma Res* 1997; **7**:S35-S42.
- 20 Hsu M-Y, Elder DE, Herlyn M. The Wistar melanoma (WM) cell lines. In: Masters JRW, Palsson B (editors): *Human Cell Cultures*, Vol. 3. London: Kluwer Academic Publishers; 1999, pp. 259-274.
- 21 Price K, Linge C. The presence of melanin in genomic DNA isolated from pigmented cell lines interferes with successful polymerase chain reaction: a solution. *Melanoma Res* 1999; **9**(1): 5-9.
- 22 Dent CL, Macbride SJ, Sharp NA, Gewert DR. Relative transcriptional inducibility of the human interferon-alpha subtypes conferred by the virus-responsive enhancer sequence. *J Interferon Cytokine Res* 1996; **16**:99-107.
- 23 Marie I, Durbin JE, Levy DE. Differential viral induction of distinct interferon- α genes by positive feedback through interferon regulatory factor 7. *EMBO J* 1998; **17**:6660-6669.
- 24 Todokoro K, Kiuoussis D, Weissmann C. Two non-allelic human interferon alpha genes with identical coding regions. *EMBO J* 1984; **3**(8): 1809-1812.