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Short Communication

α-Melanotropin Immunoreactivity in Human Melanoma Exudate is Related to Necrosis

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We have previously reported high immunoreactive α-MSH (IR-α-MSH) concentrations in melanoma patients' plasma, as well as significant amounts in melanoma metastases and cells grown in culture. Necrosis within the melanoma tumour leads to a massive proteolysis of intracellular proteins and release of cell content: this might significantly contribute to the elevated IR-α-MSH plasma levels measured in melanoma patients. To test this hypothesis, we studied the necrosis-related release of MSH from human melanoma cells, using a specific radioimmunoassay. The studies of fine-needle biopsies indicated that most of the human melanoma tumour exudates tested contained very high MSH concentrations (> 500 pg/ml; 14/15), while plasma levels were generally normal (≤ 25 pg/ml; 10/15). The level in an exudate from a non-melanoma tumour type was < 40 pg/ml. *In vitro* studies showed that release of the IR-α-MSH was time- and temperature-dependent, and related to cell death. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

α-MELANOTROPIN OR α-melanocyte-stimulating hormone (α-MSH) is a neuropeptide derived from adrenocorticotropic hormone (ACTH), a cleavage product of the larger precursor molecule pro-opiomelanocortin (POMC) [1]. POMC and its derived peptides are mainly synthesised in the pituitary, but they are also present in other normal tissues and in many tumours [2]. POMC gene expression and the occurrence of MSH-related peptides have been previously reported in human melanoma cell lines and tumours [3–7]. The melanocortins (MC; MSHs and ACTH) appear to play a key role in the regulation of proliferation and differentiation of normal and malignant melanocytes, through binding to specific G protein-coupled MC-1 receptors [8, 9].

We have found strong evidence pointing to the occurrence of a specific autocrine regulatory loop operating in human melanoma cells and involving the MC-1 receptor and an immunoreactive MSH-like peptide (IR-α-MSH) [10]. This autocrine loop might have important implications in the biology of the tumour.

In addition to an active and physiological release of IR-α-MSH by melanoma cells, necrosis occurring *in situ* within melanoma tumours might lead to massive proteolysis of intracellular proteins and release of cell content. This process might significantly contribute to the elevated IR-α-MSH levels detected in melanoma patients [3, 11, 12]. Moreover, a massive release of IR-α-MSH might lead to very high local concentrations of this peptide in the proximity of melanoma cells. This may modulate the proliferation and/or melanogenic status of the malignant cells.

To this aim, we tested whether cell death can lead to the release of IR-α-MSH from human melanoma cells, both *in situ* and *in vitro*.

MATERIALS AND METHODS

Exudate and plasma collection

Samples were obtained from 15 melanoma patients with regional (stage III) or distant metastasis (stage IV) (numbered 1–15) and from one non-melanoma patient with a

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leiomyoblastoma (16). 4a and 4b refer to different samples from the same patient. Tumour samples were obtained by fine-needle aspiration at surgery and blood samples collected at the same time; both were centrifuged.

In vitro *conditions: cell culture, incubation and viability*

The human melanoma cell line HBL (established in our laboratory) was cultured in HAM-F10 medium supplemented with 10% fetal calf serum as previously described [10]. These HBL cells were harvested by trypsin/EDTA treatment, washed 3 times with Dulbecco's phosphate-buffered saline (PBS) and maintained in suspension (50.10^6/ml) for subsequent incubations in PBS (at 37°C or 4°C, from 0 to 24 h). Cells were finally centrifuged (10 000 *g*, 10 min) to obtain supernatants for α-MSH determination. The number of viable cells was measured by the trypan blue dye exclusion method [13]. The cell pellets were further mechanically disrupted at 4°C in phosphate buffer with protease inhibitors (Complete, Boehringer), centrifuged, and supernatants were collected for MSH determination.

α-MSH radioimmunoassay (RIA)

α-MSH immunoreactivity was assessed as previously described [10]. Briefly, standard α-MSH and samples were incubated overnight with specific anti-α-MSH antiserum in phosphate buffer at 4°C. The cross-reactivity of this antiserum was below 0.001% with other POMC-derived peptides (β-MSH, γ-MSH, $ACTH_{4-10}$, $ACTH_{5-10}$, $ACTH_{17-39}$, $ACTH_{1-24}$, $ACTH_{1-39}$, β-Endorphin). In the second step, ^{125}I-NDP-MSH (^{125}I-[NLe^4,D-Phe^7]α-MSH) was added and incubated at 4°C for an additional 6 h period. The separation of antibody-bound radioactivity was achieved using charcoal/dextran procedure. All determinations were performed in duplicate, with a coefficient of variation less than 10%.

RESULTS

IR-α-MSH quantitation in human tumour exudates and corresponding plasma

α-MSH immunoreactivity was determined in tumour exudates and plasma from cancer patients. As shown in Table 1, exudates from most melanoma tumours contained very high IR-α-MSH concentrations (> 500 pg/ml for patients 1–14) when compared with the level in the leiomyoblastoma (< 40 pg/ml for patient 16). These high IR-α-MSH concentrations could also be repeatedly found in exudates from samples obtained within an interval of 2 weeks from the same melanoma patient (4a and 4b). In contrast, they were not associated with concomitant high α-MSH concentrations in plasma. These were within the normal range (≤ 25 pg/ml) for 10 out of 12 melanoma patients.

Table 1. *IR-α-MSH concentrations in both tumour exudates and corresponding plasma from melanoma and non-melanoma patients. Exudates were obtained by fine-needle aspiration at surgery and the IR-α-MSH concentrations determined by RIA*

		IR-α-MSH levels (pg/ml)	
Tissue type	Patient	Exudate*	Plasma
Melanoma	1	>1000	568
	2	>1000	25
	3	>1000	8
	4a and 4b	>1000	<6
	5 to 10	>1000	<6
	11	680	<6
	12	575	46
	13	550	<6
	14	505	ND
	15	65	ND
Leiomyoblastoma	16	35	ND

*Corresponding tumour tissue identity was confirmed by histology. ND, not done.

IR-α-MSH release associated with cell death in HBL melanoma cells

To determine whether melanoma cell death could lead to the release of IR-α-MSH, incubation experiments were performed *in vitro*, using HBL melanoma cells in suspension in a balanced salt solution without any addition of growth promoting agents. Figure 1(a) shows the MSH release kinetics both at 37°C and 4°C. We observed a significant and time-dependent increase of MSH immunoreactivity measured in the supernatants from 6 to 24 h at 37°C, while none occurred at 4°C. After 24 h of incubation, released MSH reached 80–95% of the total immunoreactivity at 37°C while it was less than 5% at 4°C (Figure 1a).

In order to investigate the mechanism involved in such a release, we evaluated cell death/necrosis under these incubation conditions using dye exclusion. The monitoring of cell death showed a decrease in cell survival mainly after 6 h at 37°C, while none was observed at 4°C (Figure 1b).

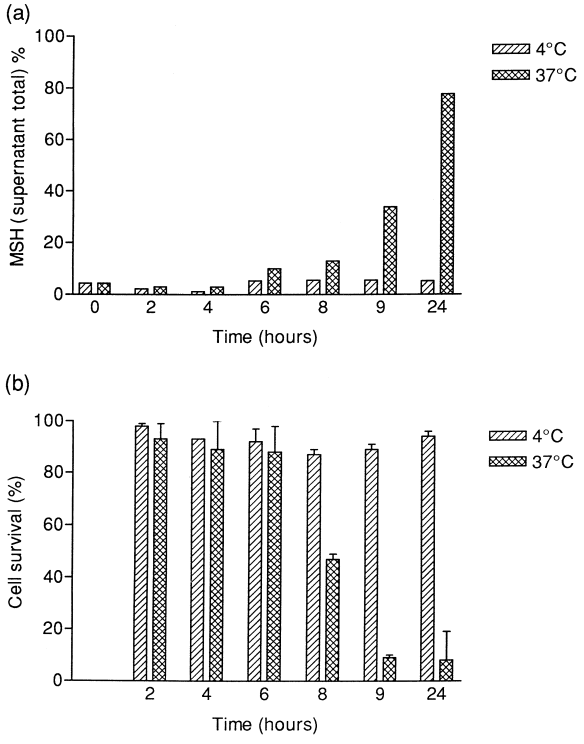

Figure 1. (a) Kinetics of IR-α-MSH release from HBL human melanoma cells and (b) cell survival in isotonic solution. Effect of temperature. Cells were incubated at 37°C and 4°C in a balanced salt solution. At different time intervals, (a) supernatants were collected, cell pellets disrupted and both submitted to α-MSH RIA. Similar trends were obtained from two independent experiments; (b) cell survival was measured by dye exclusion. Bars represent mean ± S.D.

DISCUSSION

We found that the majority of melanoma exudates had high concentrations of IR-α-MSH. Although there was no apparent correlation between exudate and corresponding blood MSH contents for most melanoma patients, patient 1 presenting an extensive cell necrosis in melanoma metastasis (determined by histology) did have an exceptionally high α-MSH plasma level. It is possible that melanoma IR-α-MSH drains to the blood. Therefore, variations in circulating levels of this peptide may occur as a result of the overall volume of exudates related to melanoma cell death and of the destruction of tumour vascular endothelium. These factors may also explain why only a third of melanoma patients display high plasma IR-α-MSH [11]. Interestingly, high levels of S-100 melanoma-associated protein were also found in melanoma exudates compared with those of non-melanoma origin and blood (data not shown). In addition, the fact that S-100 protein secretion by melanoma cells *in vitro* has not been reported, further supports the hypothesis of an *in situ* release related to melanoma necrosis. This mechanism was previously proposed by Cochran [14] for the high S-100 content in ocular fluids from patients with intra-ocular melanoma. Thus, IR-α-MSH and S-100 protein alike appear to be released by necrotic malignant melanocytes *in vivo*.

Further evidence of this necrosis-related release was displayed in a series of *in vitro* experiments, which partially mimic the *in vivo* situation except for the inflammatory process. The α-MSH release kinetic was observed at 37°C in isotonic conditions, and it was related to the monitoring of cell death. Moreover, similar release trends were also observed for S-100 protein *in vitro* (data not shown). The *in vitro* observations suggest that melanoma cell necrosis is the major mechanism involved in IR-α-MSH release for three main reasons: (1) high contents of this peptide are typical of malignant melanocytes [10]; (2) only malignant melanocytes are present in the *in vitro* experiments; (3) this release is not exclusive for α-MSH and concerns another melanoma-associated protein. Moreover, even if apoptosis occurs to some extent *in vitro*, a 'secondary necrosis' will follow. This terminal phase of *in vitro* cell death is characterised by the ultimate swelling and final lysis of the apoptotic bodies as well as remaining cell fragments [15]. However, the role and type of proteases involved in the α-MSH release during cell lysis both *in vivo* and *in vitro* need further investigations. The presence of conventional protease inhibitors acting efficiently on serine, cysteine and metalloproteases in a broad range did not affect time-dependent IR-α-MSH release at 37°C (data not shown). Therefore, this release appears to be controlled by other enzymes, most probably convertases (PC1 and PC2) reported in the pituitary to process POMC [16]. Future work will aim to specify their contribution in melanoma cells.

In conclusion, we propose that melanoma cell death might lead to the *in situ* release of large amounts of IR-α-MSH, in addition to the release associated with the autocrine loop involving IR-α-MSH and the MC-1 receptor in these cells. Accordingly, this peptide accumulates in exudates, may affect tumour biology and also subsequently be drained into the blood.

1. Eberle AN. *The Melanotropins: Chemistry, Physiology and Mechanism of Action*. Basel, Karger, 1988.
2. Bertagna X. Proopiomelanocortin-derived peptides. *Endocrinol Metab Clin North Am* 1994, **23**, 467–485.
3. Ghanem G, Verstegen J, De Rijcke S, *et al.* Studies on factors influencing human plasma alpha-MSH. *Anticancer Res* 1989, **9**, 1691–1696.
4. Lunec J, Pieron C, Sherbet GV, Thody AJ. Alpha-melanocyte-stimulating hormone immunoreactivity in melanoma cells. *Pathobiology* 1990, **58**, 193–197.
5. Ghanem G, Loir B, Hadley M, *et al.* Partial characterization of IR-α-MSH peptides found in melanoma tumors. *Peptides* 1992, **13**, 989–994.
6. Liu PY, Johansson O. Immunohistochemical evidence of α-, β- and γ3-melanocyte stimulating hormone expression in cutaneous malignant melanoma of nodular type. *J Dermatol Sci* 1995, **10**, 203–212.
7. Chakraborty AK, Funasaka Y, Slominski A, *et al.* Production and release of pro-opiomelanocortin (POMC) derived peptides by human melanocytes and keratinocytes in culture: regulation by ultraviolet B. *Biochim Biophys Acta* 1996, **1313**, 130–138.
8. Hunt G. Melanocyte-stimulating hormone: a regulator of human melanocyte physiology. *Pathobiology* 1995, **63**, 12–21.
9. Siegrist W, Eberle AN. Melanocortins and their implication in melanoma. *Trends Endocrinol Metab* 1995, **6**, 115–120.
10. Loir B, Bouchard B, Morandini R, *et al.* Immunoreactive α-melanotropin as an autocrine effector in human melanoma cells. *Eur J Biochem* 1997, **244**, 923–930.
11. Ghanem G, Lienard D, Hanson P, Lejeune F, Fruhling J. Increased serum alpha-melanocyte stimulating hormone (alpha-MSH) in human malignant melanoma. *Eur J Cancer Clin Oncol* 1986, **22**, 535–536.
12. Schwartze G, Fiedler H. Relevance of alpha-MSH plasma levels to diagnosis in human malignant melanoma. *Hautarzt* 1994, **45**, 468–470.
13. Freshney RI. *Culture of Animal Cells. A Manual of Basic Technique*. New York, Alan R. Liss, Inc., 1987.
14. Cochran AJ, Holland GN, Saxton RE, *et al.* Detection and quantification of S-100 protein in ocular tissues and fluids from patients with intraocular melanoma. *Br J Ophthalmol* 1988, **72**, 874–879.
15. Cohen JJ. Apoptosis. *Immunol Today* 1993, **14**, 126–130.
16. Seidah NG, Day R, Marcinkiewicz M, Chrétien M. Mammalian paired basic amino acid convertases of prohormones and proproteins. In Vaudry H, Eberle AN, eds. *The Melanotropic Peptides. Ann NY Acad Sci* 1993, **680**, 135–146.

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