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Lymphotoxin gene expression by melanocytes and melanoma cell lines and persistence of unspliced mRNA

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Human melanoma cell lines express many different cytokines [1], including lymphotoxin (LT), the production of which has been considered to be restricted to cells of the lymphocytic lineage in response to cell activation. LT expression by melanomas is constitutive and characterized by the presence of two mRNAs. In the present paper we report an analysis of the origin of the two LT-specific transcripts in four human melanoma cell lines at different stages of progression and in four melanocytic cell lines. Reverse transcription-polymerase chain reaction (RT-PCR) performed with primers lying in the first and fourth exons and hybridization with intron probes showed a spliced and a full-unspliced LT mRNA. This pattern was also displayed by one out of four melanocyte cell lines. Western blot analysis indicated that LT RNA is properly translated to a 23–25 kDa protein and immunocytochemistry showed its localization within the cytoplasm and on the cell membrane.

Lymphotoxin; Melanoma; RNA splicing

1. INTRODUCTION

TNF-α and -β, the latter also called lymphotoxin (LT), are two pleiotropic cytokines which share the same receptors and mediate similar functions: they are both inflammatory mediators, activate endothelial cells, granulocytes and macrophages, stimulate the proliferation and differentiation of hemopoietic cells, fibroblasts, T and B lymphocytes, and are cytotoxic for a number of tumor cells [2]. Their production has been detected in some tumor cell lines and in fresh tumor specimens [3–5]. However, while TNF-α has been described in a wide range of histological tumor types (ovarian cancer, colon carcinomas, breast and hemopoietic tumors [3–8], LT production is thought to be restricted to lymphocytes [9,10], and it has been found expressed only in tumors of the lymphoid lineage (TALL, Jurkat, Raji) [4], although its expression by different type of tumors has not been thoroughly investigated.

We have previously described that human melanoma cell lines express a wide range of cytokine, including TNF-α and LT. The endogenously produced TNF-α and LT may confer resistance to the cytotoxic effect of these cytokines given as recombinant factors [1]. LT expression detected by RT-PCR showed two bands in melanomas but only one of the two was expressed by activated lymphocytes. Here we describe the origin of the two LT transcripts in melanomas and provide evidence of the LT mature protein in these cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture and RNA extraction*

Melanoma cell lines were isolated from a primary melanoma (4405), a subcutaneous metastasis (665/1) and a local relapse of cutaneous melanoma (1402/R) from patients at the Istituto Nazionale Tumori; WM35 melanoma cell line derives from an early lesion characterized by radial growth [11]. All melanoma cells were cultured in RPMI 1640 medium supplemented with 5% or 10% FCS at 37°C in a 5% CO_2 atmosphere untill confluent. Peripheral blood lymphocytes (PBL) were purified from healthy volunteer by Ficoll gradient: 10^6 cells/ml were stimulated in vitro with 1 μg/ml of PHA for 48 h. Total RNA was extracted from cells by guanidinine isothyocyanate and purified using a cesium chloride gradient [12]. When indicated, total RNA was treated with RQ1 DNase (Promega Corp., Madison, WI, USA) for 15 min at 37°C according to the manufacturer's conditions and purified by phenol/chloroform extraction and ethanol precipitation, prior to reverse transcription. RNAs from 4 non-tumorigenic melanocytic cell cultures [13] were kindly provided by Dr. M. Herlyn (Winstar Institute, Philadelphia, PA, USA) to be analysed together with the malignant cells.

2.2. *Reverse transcription polymerase chain reaction Southern blot analysis*

One microgram of total RNA was reverse transcribed using 0.5 μg of oligo-dT primer (Clontech, Palo Alto, CA, USA) and 600 U of MMLV-RT (Gibco BRL, Gaithersburg, MD, USA) at 42°C for one hour. One μl out of 30 μl of cDNA was amplified by 30 cycles of PCR with 1 μM of each set of specific primers (Fig. 1). As control of the RT efficiency we amplified the β2 microglobulin cDNA with 15–20 cycles of PCR, to avoid reaching of the plateau of DNA synthesis; in this way we could also normalize the amount of cDNA used among the sample tested. The PCR product was run on a 2% agarose gel and transferred to nylon filters (Hybond Amersham, Buckinghamshire, England, UK) by alkaline blotting [12]. The blots were hybridized with a full-length LT cDNA probe derived from PHA-stimulated PBL or with two synthetic oligonucleotides complementary to sequences in the second and third intron of the LT gene.

Genomic DNA was extracted from melanomas and PHA-stimulated PBL as described [14]: 5 μg of DNA were digested with *Ava*I,

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*Bam*HI, *Eco*RI, *Pst*I and *Pvu*II restriction enzymes (New England Biolabs Inc., Beverly, MA, USA) according to the manufacturer's conditions, electrophoresed on 0.8% agarose gel and transferred on nylon filters, which were then hybridized with the full length LT cDNA probe. After stringent washing the filters were exposed for autoradiography using intensifying screens.

2.3. *Western blot analysis and immunocytochemistry*

Melanoma cells and PHA-stimulated PBL were lysed in 10 mM Tris-HCl, 0.15 M NaCl, 1% NP40 containing 1 μg/ml aprotinin and leupeptidin and 100 μg/ml phenylmethylsulphonyl fluoride (PMSF). The protein content of the cell extracts was measured using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and equal amounts of protein were separated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA) by electroblotting [12]. Immunodetection of LT was performed by overnight incubation of the filter with 10 μg/ml of goat-anti human LT (British Bio-technology Ltd., Cowley, Oxford, UK) at 4°C. After extensive washing in 10 mM Tris-HCl, 0.15 M NaCl the filter was incubated with 0.22 μCi/ml of [^{125}I]protein G (Amersham, Buckinghamshire, UK) for 1 h at room temperature, repeatedly washed, dried and autoradiographed for 7 days. For immunocytochemical analysis, 25×10^3 melanoma cells or PHA-stimulated PBL were cytocentrifuged on glass slides and fixed in cold 4% buffered paraformaldehyde for 5 min. The slides were incubated overnight with diluted rabbit-anti human LT polyclonal antibody (Endogen Inc., Boston, MA, USA) at 4°C; then the slides were sequentially treated with mouse anti-rabbit and rabbit anti-mouse immunoglobulins, followed by a mouse-APAAP conjugate (Dako A/S, Glostrup, Denmark); this treatment was repeated twice before addition of the chromogenic substrate for the alkaline phosphatase reaction.

3. RESULTS

3.1. *LT transcription in melanomas and melanocytes*

Nineteen melanoma cell lines were previously screened for expression of LT RNA [1]. RT-PCR, performed with commercial primers (primers 1 and 4, Fig. 1), showed LT transcription that was characterized by two bands in 13 out of 19 lines, while PBL RNA, used as a positive control, displayed a single band. Three melanoma cell lines (665/1, 1402/R and 4405) were used to further investigate the origin of the double band pattern, that might result from a gene rearrangement or represent the expression of an alternatively spliced mRNA or simply derive from a DNA contamination of the RNA preparation. DNA rearrangement is unlikely because Southern blot analysis showed a restriction enzyme pattern that was identical in the DNA of the three melanoma cell lines as well as in the DNA from PHA-stimulated PBL, digested with five different endonucleases which do not detect genetic polymorphism (not shown).

RT-PCR performed with a different set of primers allowing the amplification of the entire LT open reading frame, thus including the four exons and the three introns (Fig. 1A,B: primers 3 and 2), confirmed the double band pattern in melanomas but not in activated

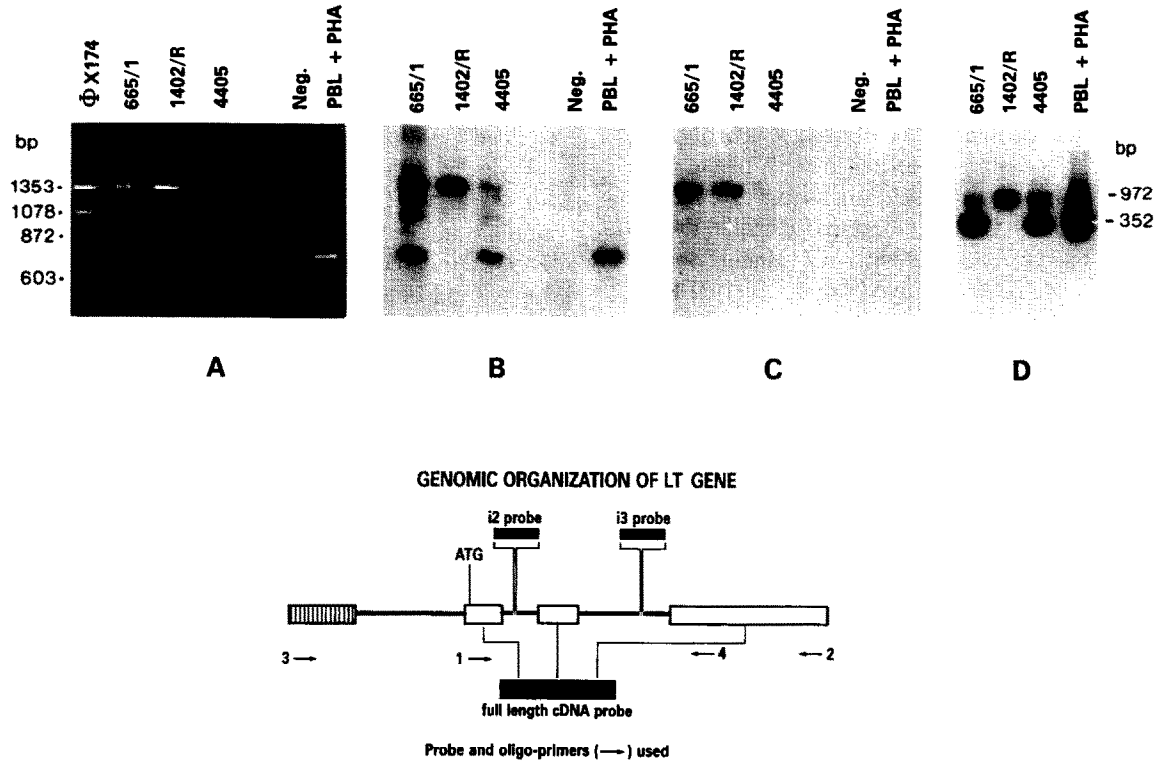

Fig. 1. RT-PCR analysis of LT expression in human melanomas and schematic representation of the LT gene. Amplification with primers 2 and 3 reveals the presence of an unspliced mRNA in melanomas but not in PHA-stimulated PBL, detectable both in an ethidium bromide-stained gel (A), and after hybridization with a full-length cDNA probe (B); hybridization with an oligonucleotide probe specific for intron 2 shows the persistance of unspliced LT mRNA in melanomas (C). DNase treatment of RNA before reverse transcription and amplification with primers 3 and 4 confirmed that the two LT bands derive from specific transcripts and not from DNA contamination (D).

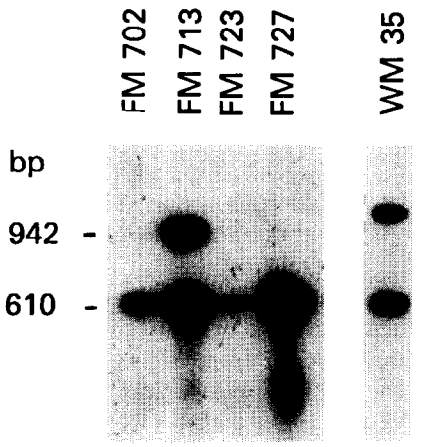

Fig. 2. LT expression in four melanocyte cell lines and in the 'radial growth phase' melanoma WM35, as analysed by RT-PCR performed with primers 1 and 2 (Fig. 1) and followed by hybridization with the full-length cDNA probe.

PBL. Band sizes were compatible with a transcript containing all three introns and with a fully spliced mRNA. Hybridization with oligonucleotide probes complementary to the second (Fig. 1C) and third intron (not shown) confirmed this hypothesis. Finally, DNA contamination of the starting RNA was excluded by treating the total RNA with DNase before reverse transcription and by finding the double band pattern in melanomas but not in PBL RNA after amplification with primers 1 and 4 (Fig. 1D).

In order to see whether spliced and unspliced LT RNA are related to either a different degree of tumor progression or simply to malignant transformation, RT-PCR was performed on additional RNA obtained from the WM35 cell line, a radial growth-phase melanoma, and from four foreskin melanocyte cell lines (kindly provided by Dr. M. Herlyn). Results showed that all samples expressed LT with WM35 and one out of four melanocyte lines displayed the doublets (Fig. 2).

3.2. *LT production and cellular localization*

In spite of mRNA expression, LT was undetectable by ELISA in the supernatant of Me 665/1, 1402/R and 4405 [1]. We therefore tested by Western blot and immunocytochemistry whether these melanomas may synthesize LT. Immunoblotting revealed a specific band of 23–25 kDa compatible with a monomeric protein of molecular size expected from the spliced mRNA (Fig. 3).

Immunocytochemistry performed on the same melanoma cell lines showed that LT localizes within the cytoplasm and on the cell membrane of the majority of the cells (> 95%) (Fig. 4).

4. DISCUSSION

Expression of LT in B and T lymphocytes is tightly regulated and normally repressed [15,16]. Its expression is induced by different signals, including antigens, mitogens, phorbol esters and cytokines, such as IL-2 and IL-1β [10,17–20] in both normal lymphocytes and tumor cells of lymphoid origin [4,21]. Constitutive expression of LT has been rarely reported and confined to lymphomas [22], multiple myelomas [23], and to virally transformed lymphoid cells, such as B lymphoblastoid cell lines, Raji cells [4,24] and HTLV-I infected tumor cell lines [25]. We have previously detected constitutive expression of LT in most of the melanoma cell lines analysed by RT-PCR [1]. Here we demonstrate that of the two specific LT transcripts one represents the unspliced mRNA, containing all the three introns, and that the other is the fully spliced mRNA.

In the murine T cell line CTLL-2, LT expression is induced by IL-2 and is regulated through the alternative splicing of an immature polyadenilated mRNA that contains all three introns [26] and that gives rise to three different mRNAs. A similar mechanism of posttranscriptional regulation could be responsible for the double bands detected by RT-PCR in our melanomas, although we could not detect any alternatively spliced mRNA, but only an immature and fully spliced transcript. As for CTLL-2 cells [26], however, in human melanomas the polyadenilation of the LT transcript occurs before the intron splicing, therefore both mRNAs could be reverse transcribed using a poly-dT primer.

Many other genes appear to be regulated at posttranscriptional level by either alternative intron splicing, as reviewed in [27], or by ordinate removal of all intron sequences, as for thymidine kinase or phosphoenolpyruvate carboxykinase [28,30]; in these cases unspliced, spliced and partially spliced mRNAs are detectable. Furthermore, the murine lymphoma cell line SL12.4 shows a posttranscriptional regulation of the TCR-α and -β chain expression with accumulation of unspliced RNA. To detect the spliced form, an appropriate stimulus should be given: in such a case both unspliced and

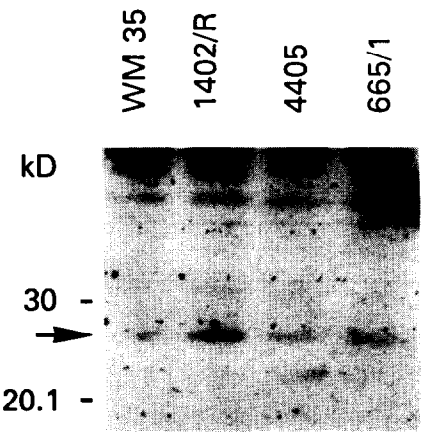

Fig. 3. LT production by human melanomas, analysed by immunoblotting. Equal amounts of protein were loaded per lane and separated on 15% SDS-PAGE. Arrow indicates the 23–25 kDa specific band detected with anti-human LT antibody.

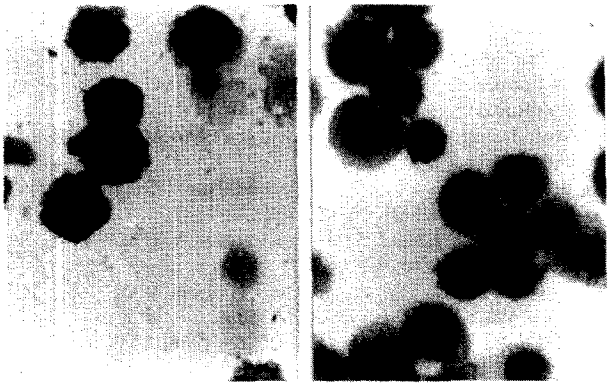

Fig. 4. LT detection by immunocytochemistry. Immunocytochemistry was performed using the APAAP technique on melanoma cells and showed LT-positive staining in the cytoplasm and on the cell membrane. 665/1 (right) and 1402/R (left) cell lines are shown as representative of the melanomas analysed.

spliced mRNAs become detectable within these cells [30,31]. This pattern of gene expression in SL12.4 lymphoma is most similar to that occurring for LT in the melanomas analysed in this study, since both show the persistance of the unspliced and the fully spliced RNAs. What is different between these two tumors is that SL12.4 cells display the spliced RNA only after appropriate treatment, while in melanomas the two RNAs seem constitutive. Without success we tried to modulate LT expression by treatment with TNF-α and IFN-γ given, alone or in combination, at doses known to modulate the expression of various molecules, such as integrins, tumor-associated antigens and HLA class II antigen on melanomas [32].

Normal PBL stimulated with PHA expressed only the mature form of LT mRNA, suggesting that in melanomas its expression might be related to the malignant phenotype and that the anomalous two-band pattern may be linked to tumor progression. Southern blot analysis rejected the hypothesis of a rearrangement in the LT gene and RT-PCR revealed that LT was indeed expressed in four melanocyte cultures, one of which displayed the unspliced mRNA also. This different RNA pattern might represent a peculiar posttranscriptional regulation occurring in cells of the melanocytic lineage or might indicate an impairment of the RNA splicing machinery in the melanoma cells. The analysis of four melanocytic cell lines may be insufficient to conclude that the double band pattern is restricted to the transformed phenotype; the finding in one out of four melanocyte cultures of the unspliced LT mRNA band does not rule out this hypothesis. It should be considered that melanocytes are kept in culture with promoting agents like TPA [33] and, although other phenotypic markers are compatible with a non-transformed state, these growth conditions might interfere with some biological function.

Although the secreted form of LT was undetectable by ELISA assay [1], LT detection was possible by Western blot and immunocytochemistry, thus suggesting that its localization was confined to the cytoplasm and the cell membrane.

The presence of a single LT specific band of 23–25 kDa in Western blots indicates that the immature RNA could not be translated to give a new protein, as was suggested by Weil for the murine LT mRNAs obtained from the CTLL-2 cell line [26]. However we could not exclude the possibility that a putative alternative protein might exist but lacking the epitopes recognized by our antibody. While the relative amount of the two LT mRNA forms is different among the melanoma cell lines (Fig. 1), the presence of a LT monomeric protein of the correct size, particularly in the 1402/R cell line that mostly expresses the unspliced mRNA form, suggests that splicing occurs in all the cell lines thus giving rise to a functional mRNA.

The functional role of LT expression by melanoma cells remains unknown. Easier speculation might be possible if a soluble protein could have been detected, thus allowing to consider all the LT paracrine effects, for example its osteolytic and bone resorption activity that might facilitate tumor spreading to bones [23,24]. In addition, LT has been described to be cytotoxic for the same cells producing it, as for stimulated B and T lymphocytes [35]. This observation suggested a role for LT in immunoregulation and ontogeny of B and T lymphocytes [9,18,36] but may also suggest a possible mechanism of tumor-derived immune-suppression. Finally, LT production even if confined to the membrane and cytoplasm of tumor cells, might represent a mechanism of escape from the cytotoxic effects of T cell-produced LT and of monocyte-produced TNF-α [36,37].

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