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ORIGINAL ARTICLE

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Expression of the endothelin-B receptor in pigment cell lesions of the skin

Evidence for its role as tumor progression marker in malignant melanoma

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Abstract Endothelins (ETs) exert several functions in human melanocytes, including proliferation, dendrite formation, and melanin synthesis. Among the ET receptors, the non-selective endothelin-B (ETB) receptor is the major receptor in melanocytes and malignant melanoma (MM) cells. In spite of the important role of ETs and their receptors in the growth and differentiation of melanocytes, the distribution and expression levels of ETB receptors in tissue sections of benign and malignant pigment cell lesions is still unknown. We combined immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) to study ETB receptor expression in benign and malignant pigment cell lesions and in normal skin. Immunohistochemistry on paraffin-embedded tissue sections of 159 cases revealed a significant increase in intensity of ETB receptor expression from common nevi over dysplastic nevi and primary MM to metastatic MM. Quantitative PCR using real-time detection on 75 samples confirmed the immunohistochemical results. These data add the ETB receptor to the growing list of tumor progression markers in MM and suggest that ETs play a role in the progression of MM in the skin.

Keywords Melanoma · Melanocyte · Endothelin · Receptor · Tumor progression

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Introduction

Endothelins (ET-1, ET-2, ET-3) are paracrine signal peptides with 21 amino acid residues binding to at least two subtypes of G protein-coupled heptahelical receptors, termed endothelin-A (ETA) and endothelin-B (ETB) receptor, which were originally isolated from cultured porcine aortic endothelial cells [7, 31]. The two receptors share high sequence homology but can be distinguished by their specificity, i.e., a selective ETA receptor that binds ET-1 and ET-2 with high affinity but ET-3 with low affinity and a non-selective ETB receptor that binds all ET isopeptides with equal affinity [9, 17].

The ETs are particularly known for their endothelial localization and vasoconstrictive effects [20, 31], but recent data suggest that they exert additional physiological functions [26]. ETs are believed to play an important role in melanocyte development [33], including the regulation of melanocyte progenitor cell number, and the induction of pigment synthesis [25] and dendrite formation in these cells [11]. These findings suggest that ETs stimulate differentiation of the melanocyte progenitors into fully mature melanocytes. ETs also serve as mitogen for melanocytes and stimulate DNA synthesis of primary and recurrent cutaneous melanoma cells in serum-deprived cultures. In this respect, ETs are equipotent to basic fibroblast growth factor (bFGF) and are co-mitogenic with bFGF in an additive manner for human melanocytes [17]. ETs stimulate human melanocyte proliferation and differentiation via a receptor-mediated signal transduction pathway [30], and it has recently been demonstrated that the ETB receptor is the major receptor involved in the ET-1-induced differentiation of melanocytes [17].

In view of the mitogenic effects of ETs for various cell types in vitro [3] and in vivo [15], the ETB receptor has extensively been studied in several cancers [13, 32]. Mutations or altered expression of this receptor have been observed in prostate and breast cancer [2, 18, 22]. The distribution and expression of ETB receptors in pigment cell lesions has not been studied in vivo. Therefore, we analyzed the expression of the ETB receptors in a

large series of benign and malignant pigment cell lesions using immunohistochemistry and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. We observed a gradual increase in ETB receptor expression on both the protein and RNA level from common nevus to metastatic malignant melanoma (MM), suggesting that ETB receptors and, hence, ETs, play a role in the tumor progression of MM.

Materials and methods

Materials

For immunohistochemistry, 159 buffered formalin-fixed paraffin-embedded benign and malignant pigment cell lesions were used. These included 61 benign lesions, 15 nevi with varying degrees of architectural, cytological, and stromal dysplasia assessed according to standard criteria [8], 10 MM in radial growth phase (RGP) only, 56 MM in vertical growth phase (VGP), and 17 metastatic MM. The diagnoses of the benign nevi are listed in Table 1.

For RT-PCR, we used frozen tissue from 75 samples, including 9 cases of normal skin, 16 common nevi, 25 primary MM, and 25 metastatic MM. As a positive control in immunohistochemistry and RT-PCR, tissue from normal adrenal gland was used since this tissue has been shown to express large quantities of the ETB receptor [13].

Methods

In preliminary experiments comparing various immunohistochemical procedures and antigen retrieval methods, the Dako EnVision System proved to be the most sensitive for the demonstration of ETB receptors in paraffin-embedded material. Incubation with polyclonal rabbit anti-ETB receptor antibody (kindly provided by T Suzuki, Fukushima, Japan) for 30 min at room temperature was followed by peroxidase-conjugated EnVision+ reagent (Dako, Copenhagen, Denmark) for another 30 min. The anti-ETB receptor antibody was raised against a 14-mer peptide of human ETB receptor (positions 420–433 amino acid residues) and identifies a 51-kDa protein on Western blotting [13]. Enzyme activity was developed using 3-amino-9-ethylcarbazole and H_2O_2, revealing a bright red color that contrasted well with the brown melanin pigment. Controls in which the primary antibody was replaced by phosphate-buffered saline (PBS) did not reveal specific staining. In five cases in which the rabbit antibody showed clear-cut immunoreactivity, serial sections were stained with a commercially available sheep anti-ETB receptor antibody (Oxford Biomedical Research) and yielded similar, albeit much weaker results.

Only cases in which at least 10% of the neoplastic cells in a given tumor progression phase (i.e., RGP, VGP, or metastatic phase) reacted were considered positive; in these cases, the intensity of cytoplasmic staining was semiquantitatively assessed in each growth phase by two of the authors (AD and JJvdO) as (+), weak immunoreactivity; 1+, moderate immunoreactivity; and 2+ for strong immunoreactivity. In addition to cytoplasmic staining, nuclear immunoreactivity also frequently occurred but was not evaluated semiquantitatively (Table 1). In cases of discrepant scoring, agreement was reached upon discussion. For statistical analysis, the Mann-Whitney test was used with a significance level of less than 5%.

For RT-PCR, total RNA was extracted from ten sections of 20-µm thickness in all 75 cases using Trizol reagent (Life Technologies, Merelbeke, Belgium). Total RNA (2 µm) was converted into complementary DNA (cDNA) using superscript reverse transcriptase according to the manufacturers recommendations (Life Technologies). The RT-PCR for the constitutively expressed Forkhead transcription factor (FKHR) gene at chromosome 13 served as a control test for the mRNA quality.

Table 1 Results of immunohistochemistry in benign and malignant melanocytic (MM) lesions. Immunohistochemistry was performed in 159 cases. The exact number of each group is given in column 2 (*n*). Clark level I and lentigo maligna (LM) are grouped. For each pigment cell lesion, the junctional and dermal compartments were investigated separately. Cytoplasmic staining (C) was graded as (+) (weak), + (moderate) and ++ (strong)

Diagnosis	*n*	Junctional						Dermal					
		n	Negative	Positive	C(+)	C+	C++	*n*	Negative	Positive	C(+)	C+	C++
Common acquired nevi[a]	26	19	9 (47%)	10 (53%)	10/10	–	–	14	8 (57%)	6 (43%)	6/6	–	–
Other benign lesions[b]	21	21	9 (43%)	12 (57%)	5/12	4/12	3/12	13	5 (38%)	8 (62%)	5/8	2/8	1/8
Common blue nevi	6	0	–	–	–	–	–	6	4 (66%)	2 (34%)	–	2/2	–
Cellular blue nevi	8	0	–	–	–	–	–	8	3 (38%)	5 (62%)	–	1/5	4/5
Dysplastic nevi[c]	15	15	5 (33%)	10 (67%)	3/10	5/10	2/10	6	5 (83%)	1(16%)	–	–	1/1
MM CI AND LM	10	10	5 (50%)	5 (50%)	2/5	2/5	1/5	0	–	–	–	–	–
MM Cl II	13	13	1 (8%)	12 (92%)	6/12	4/12	2/12	13	6 (46%)	7 (54%)	4/7	3/7	–
MM Cl III	20	19	2 (10%)	17 (90%)	3/17	8/17	5/17	20	7 (35%)	13 (65%)	5/13	4/13	4/13
MM IV	15	13	3 (23%)	10 (77%)	3/10	4/10	3/10	15	3 (20%)	12 (80%)	2/12	4/12	6/12
MM V	8	5	0 (0%)	5 (100%)	4/5	1/5	–	8	3 (37%)	5 (63%)	2/5	1/5	2/5
Metastases	17	0	–	–	–	–		17	0 (0%)	17 (100%)	7/17	5/17	5/17

[a] Five lentigines, six junctional nevocellular nevi (ncn), six compound ncn, and nine dermal ncn

[b] Three halo nevi, four junctional and eight compound Spitz's nevi, and four junctional and two compound Reed nevi

[c] Nine junctional and six compound dysplastic nevi

Table 2 Oligonucleotide primer and probe sequences used

Gene	Oligonucleotide	Sequence	Product size
ETB rec[a]	Forward primer	5'-TGAGTCTATGTGCTCTGAGTATTGACA-3'	
	Reverse primer	5'-ACCTATGGCTTCAGGGACAGC-3'	149
	Probe	5'-TGTTTTGATTTGGGTGGTCTCTGTGGTTCT-3'	
GAPDH[b]	Forward primer	5'-AGCCTCAAGATCATCAGCAATG-3'	
	Reverse primer	5'-ATGGACTGTGGTCATGAGTCCTT-3'	101
	Probe	5'-CCAACTGCTTAGCACCCCTGGCC-3'	

[a] Reverse transcriptase polymerase chain reaction analysis was done for the endothelin-B receptor (ETB rec), revealing an amplicon of 149 bp. A FAM (6-carboxy-fluorescein)-labeled probe was used

[b] The endogenous control, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), had an amplicon length of 101 bp. A JOE-labeled probe was used

Real-time RT-PCR

Theoretical basis

This procedure is based on the time point during cycling when amplification of the PCR product is first detected, rather than by the amount of PCR product accumulated after a fixed number of cycles. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above the baseline [12]. The ETB receptor target gene copy number in unknown samples is quantified by measuring Ct and by using a standard curve to determine the starting copy number.

A standard curve was constructed for ETB and for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, as an endogenous control. Serial dilutions (in triplicate) of total RNA from normal adrenal gland were made in diethylpyrocarbonate (DEPC)-treated water with 1000, 500, 200, 100, 50, 20, and 10 ng of normal adrenal gland. The series of diluted human total RNAs were aliquoted and stored at –80°C until use. RNA was treated with DNase (Gibco Life-technologies) to prevent the amplification of possible pseudogenes.

The target amount of the unknown samples were divided by the endogenous reference amount to obtain a normalized target value. Final results, expressed as *n*-fold differences in ETB receptor gene expression relative to the *GAPDH* gene and the calibrator were calculated as described previously [4].

All PCR reactions were performed in an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The reaction mixture contained the *Taq*man universal master mix (Perkin-Elmer Applied Biosystems, Part no.°4304447), 1 µl cDNA, 15 pmol each primer, and 10 pmol FAM (6-carboxy-fluorescein)-labeled ETB receptor *Taq*man probe (Eurogentec, Seraing, Belgium; Table 2). The thermal cycling conditions comprised an initial denaturation step at 94°C for 10 min and 40 cycles at 94°C for 20 s and at 60°C for 1 min. Experiments were performed in triplicate for each data point. Each PCR run included the seven points of the standard curve (a set of three serially diluted normal human adrenal gland cDNAs), a no-template control, and the unknown cDNAs.

Results

Immunohistochemical findings

In normal skin, the ETB receptor was expressed in keratinocytes, the eccrine sweat glands and, variably, in scattered macrophages. Except for blue nevi, 53% of the 26 common acquired lesions showed immunoreactivity in their junctional component and 43% in the dermal component. The intensity of staining in the dermal component was usually less than that of the junctional component (Table 1). Two of six common blue nevi showed cytoplasmic immunoreactivity, in contrast to cellular blue nevi, of which 62% showed immunoreactivity, which was more intense than that of common blue nevi (Fig. 1a).

The dysplastic nevi in this study comprised nine junctional and six compound lesions. The junctional component of 10 of 15 (67%) showed strong immunoreactivity (Fig. 1b), whereas the dermal component was positive in only one out of six cases. Of 15 dysplastic nevi, 7 also showed nuclear immunoreactivity. Dysplastic nevi and common acquired nevi did not differ significantly in intensity of junctional ETB receptor expression.

Expression of the ETB receptor in MM increased with increasing level of invasion. Whereas half of the in situ or Clark I MM, including lentigo maligna, expressed the ETB receptor (Fig. 1c), all Clark V MM expressed the ETB receptor. With respect to the immunoreactivity in the distinctive phases of tumor progression, it was evident that the staining intensity in the intra-epidermal component (i.e., the radial growth phase) did not differ significantly between MM of various invasion levels, whereas the intensity of staining in the invasive (dermal) component increased with successive Clark levels. Thus, in all Clark-II and -III MM, the ETB receptor expression in the in situ component was more intense or equal to that of the invasive part of the tumor (Fig. 1d), whereas in 8 of 23 Clark-IV and -V MM, the reverse was observed, i.e., a more intense immunoreactivity in the dermal component than in the epidermal part. Nuclear reactivity was seen in 40–60% of all types of MM.

Comparing the intensity of staining in nevi with that in primary MM, no statistically significant differences were seen between dysplastic nevi and Clark I/II MM, whereas Clark-III (P=0.0399) and Clark-IV (P=0.0101) MM showed a significantly more intense staining in their dermal component than dysplastic nevi.

All metastatic melanomas showed cytoplasmic reactivity and half of them revealed additional nuclear staining. Immunoreactivity was frequently more intense at the periphery and diminished towards the center of the metastasis (Fig. 1e). In involved lymph nodes, the subcapsular metastatic MM cells were more intensely stained than those within the lymph node parenchyma.

For the evaluation of the immunohistochemical expression of the ETB receptor throughout the tumor pro-

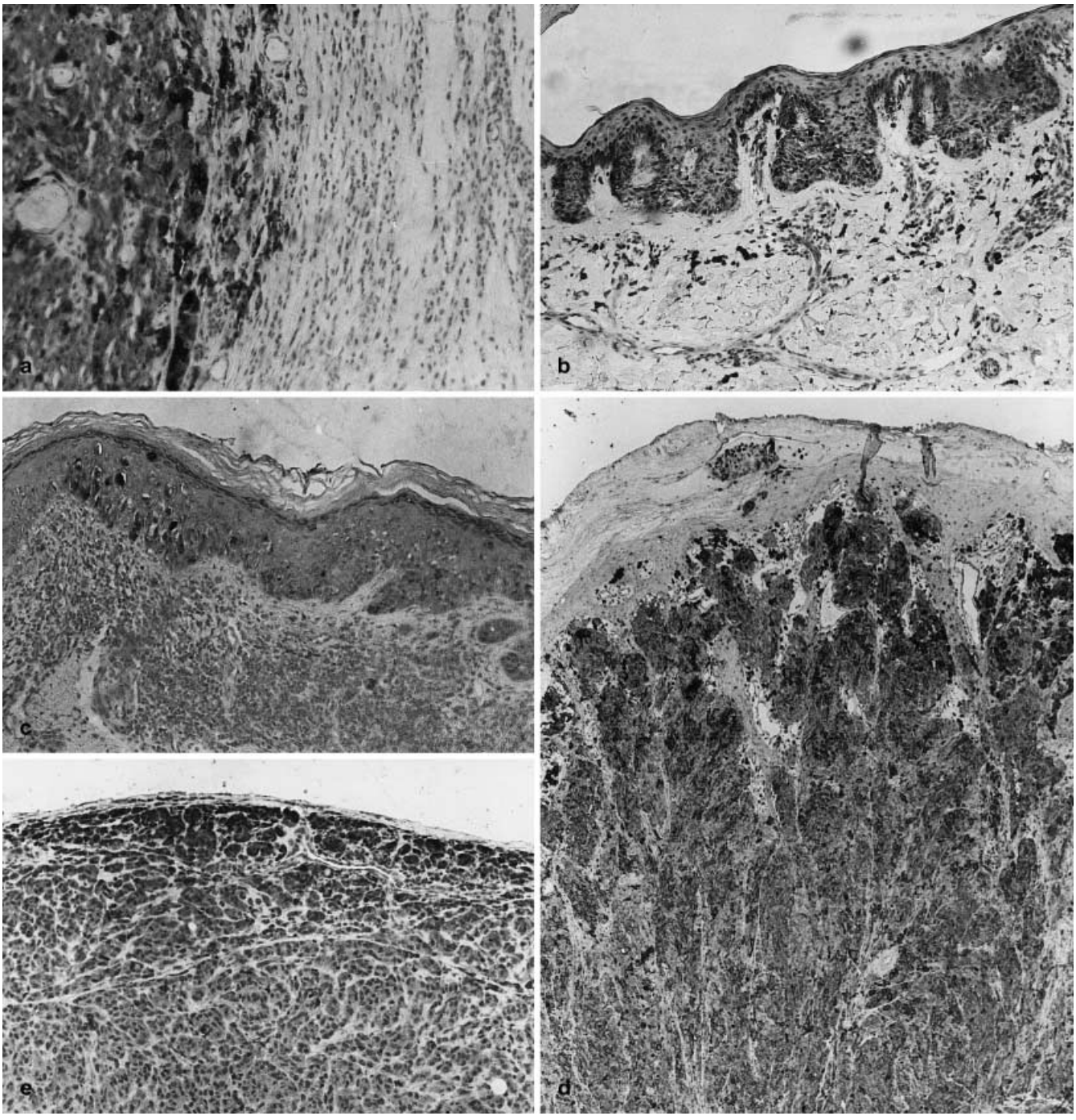


Fig. 1 Endothelin-B (ETB) receptor expression in pigment cell lesions of the skin. **a** Combined nevocellular nevus, composed of a (unstained) common nevocellular nevus on the *right*, and an immunoreactive cellular blue nevus on the *left*. **b** In this junctional dysplastic nevocellular nevus, strong immunoreactivity occurs in the lentiginous parts of the lesion. There is variable nuclear staining in keratinocytes. **c** Clark-I superficial spreading malignant melanoma (MM), showing strong immunoreactivity in Pagetoid neoplastic cells in the epidermis. **d** Clark-IV acrolentiginous MM, showing intense, membranous staining in tumor cell nests at the junction, whereas immunoreactivity gradually decreases towards the reticular dermis. **e** Metastatic MM in the skin, showing diffuse immunoreactivity that is more pronounced at the edge of the lesion. Three-step avidin–biotin peroxidase complex (ABC) technique, rabbit anti-ETB receptor antibody, counterstained with hematoxylin, ×218 (**a**) and ×87 (**b**–**e**)

gression phases of MM, four major groups were compared, i.e., common acquired nevi, dysplastic nevi, primary MM, and metastatic melanomas (Fig. 2). No significant difference in intensity of immunoreactivity was seen between common and dysplastic nevi. However, significant differences in intensity of staining were found between common acquired nevi and primary MM (P=0.0005 for the epidermal part and P<0.0001 for the dermal component), between dysplastic nevi and primary MM (P=0.0221 for the dermal compartment) and between primary MM and metastatic melanoma (P=0.05).

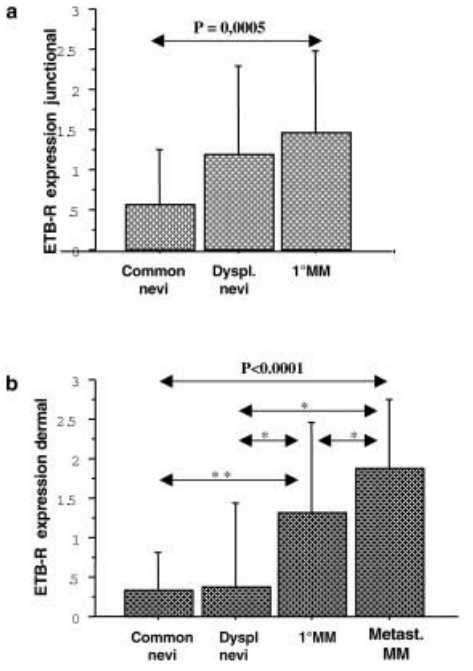


Fig. 2 Increased endothelin-B (ETB) receptor expression in malignant melanoma (MM) and their metastases versus normal and dysplastic nevocellular nevi. The immunohistochemical results on 159 melanocytic samples are shown in the junctional (**a**) and dermal (**b**) part of the pigment cell lesion. Intensity of the staining was divided into three groups as described in Table 1. The Mann Whitney test was used for the statistical analysis (**P*<0.05; ***P*<0.01). *Bars* mean±SD. No significant difference was seen between common acquired nevi and dysplastic nevocellular nevi (*Dyspl. Nevi*). Differences between the other groups are shown. A highly significant difference was found between primary melanoma (*1_MM*) and metastatic MM (*Metast. MM*)

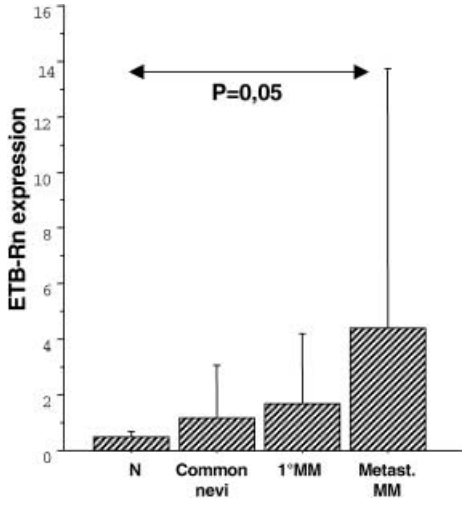


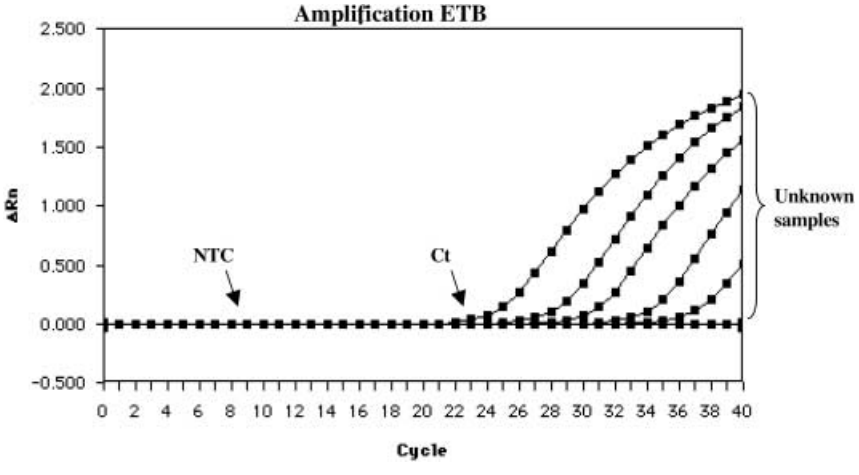
Fig. 4 Real-time detection of endothelin-B (ETB) receptor expression levels in melanocytic lesions versus normal skin samples. RNA from 75 samples was extracted and was used for reverse transcriptase polymerase chain reaction (RT-PCR), as described in Materials and methods. Normal skin samples (*N*) were also included to compare the normalized ETB receptor expression (*ETB-Rn*) levels in normal tissue and pigment cell lesions. Three groups were investigated: common nevocellular nevi (*common nevi*), primary melanoma (*1_MM*) and metastatic MM (*Metast. MM*). Statistical analysis was performed using the Mann Whitney test. *Bars* mean±SD

Reverse transcriptase polymerase chain reaction

Real-time RT-PCR was performed on 9 normal skin samples, 16 nevi, 25 primary MM, and 25 metastatic melanomas. In five cases, both the primary MM and the metastasis from the same patient could be evaluated. Figure 3 shows a real-time PCR standard curve for the ETB receptor gene. A strong linear relationship between the Ct and the log of the starting copy number was always obtained. The efficiency of the reaction (E), calculated by the formula: E=10^1/|m|-1, where *m* is the slope of calibration curve, was 90–100%.

Normalization of the 75 amplified samples according to their *GAPDH* gene expression (Fig. 4) revealed significant differences in level of ETB receptor expression between normal skin (mean 0.470±0.067) and metastatic melanoma (mean 4.380±1.874; *P*=0.05). A trend toward significance was found between normal skin and primary MM (mean 1.665±0.505), whereas no difference in level of ETB receptor expression was found between normal skin and nevi. When normal skin and nevi (mean 1.151±0.482) were taken together, they showed a signifi-

Fig. 3 Endothelin-B (ETB) receptor expression in malignant melanoma using real-time polymerase chain reaction (PCR). Amplification plots for reaction with the ETB gene and a no template control (*NTC*). Cycle number is plotted versus change in normalized reporter signal (*Δ Rn*). For each reaction tube, the fluorescence signal of the reporter dye (*FAM*) is divided by the fluorescence signal of the passive reaction dye (*ROX*) to obtain a ratio defined as the normalized reporter signal



cantly lower ETB receptor expression than the group of malignant pigment cell lesions ($P<0.05$). The range of results observed in metastatic melanomas was wider than that observed in primary MM, nevi, and normal skin.

Discussion

Using immunohistochemistry and RT-PCR, we have studied the expression of the ETB receptor in a large series of benign and malignant pigment cell lesions. In order to elucidate the possible role of ETB receptors in the progression of MM in vivo, we used biopsy material for both the immunohistochemistry and RT-PCR, and we analyzed both benign and malignant pigment cell lesions. Both cytoplasmic and nuclear immunoreactivity were observed. Although as yet unexplained, nuclear immunoreactivity for the ETB receptor has previously been described in other tissues [13], this study is the first in which ETB receptor levels in benign nevi have been analyzed. Although cytoplasmic immunoreactivity occurred in the dermal component of almost half of benign nevi, RT-PCR revealed that these nevi did not differ significantly from normal skin samples, indicating that common nevus cells express only low levels of the ETB receptor. In contrast, ordinary and cellular blue nevi showed enhanced immunoreactivity for the ETB receptor. This feature may be related to the increased melanin formation in these cells which, in turn, may reflect an increased response to ET-1, a well-known inducer of melanin [25]. The strong ETB receptor expression in blue nevi may also point towards their close relationship with migrating melanocyte precursors that express high levels of the ETB receptor [24].

Neither immunohistochemistry nor RT-PCR could detect significant differences in the level of ETB receptor expression between common nevi or normal skin and dysplastic nevi, although a slight upward trend was evident (Fig. 2). These data suggest that associated stromal cells contribute little if any to the ETB receptor expression. In contrast, immunohistochemistry showed that primary MM exhibited a more intense ETB receptor immunoreactivity than dysplastic nevi, whereas metastatic melanomas in turn showed a remarkably increased staining intensity relative to primary MM. Within the scheme of tumor progression in the melanocytic system [6, 10], these data thus show an increased ETB receptor expression with successive phases of tumor progression and suggest that ETB receptors and hence ETs are involved in the tumor progression of MM. Although comparative statistical analysis revealed significant differences in our cases, studies analyzing larger groups should be performed to confirm these findings.

Based on our data, we can speculate on the role of ETB receptors in the progression of MM. The mechanism by which neoplastic cells in advanced phases of tumor progression upregulate their ETB receptor expression remains to be studied, but a parallel may be drawn with the pathway of the MM progression marker interleukin (IL)-6, in which both autocrine and paracrine mechanisms are operative, with ultra violet (UV) radiation as an important trigger. The production of IL-6, a well-known tumor progression marker in MM, is upregulated by keratinocytes under influence of UVA [5, 16]. Apart from this paracrine mechanism (keratinocyte-derived IL-6), dermal MM cells in thick melanomas not only express IL-6 receptors but also produce IL-6 in an autocrine way [21]. This dermal autocrine loop of IL-6 production and IL-6 receptor expression appears to be closely associated with increasing growth autonomy of the malignant cells.

A similar model can now be suggested for ET-1 and the ETB receptor in MM. Although ET-3 also utilizes the ETB receptor, we believe this growth factor to be of less importance in MM progression since it is not upregulated by UV light [14, 25]. Previous studies reported the UV light-induced synthesis of ET-1 by keratinocytes [1, 14, 15, 16, 28]. UVB-induced ET-1, derived from these keratinocytes, acts as a paracrine factor for melanocyte growth and melanization and as an autocrine growth factor for keratinocytes [14]. Moreover, ET-1 upregulates ETB receptors on keratinocytes [28]. Hence, melanoma cells in the radial growth phase are likely to be activated mainly by keratinocyte-derived ET-1. In the dermal component of MM, two pathways may be operative. One pathway might involve an autocrine loop in which MM cells produce ET-1, express ETB receptors, and upregulate their own ETB receptors by ET-1, similar to epidermal keratinocytes. Alternatively, a paracrine mechanism might be operative in the invasive part of MM and involve stimulation of MM cells by ET-1 derived from endothelial cells.

Previous studies compared the ETB receptor expression in cell lines derived from primary and metastatic MM and found decreased expression levels in metastatic cell lines [7, 17]. They attributed this downregulation of the ETB receptor to tumor progression resulting in reduced apoptosis of melanoma cells by ET-1, although others found that ET-1 protects against apoptosis in a variety of tissues [23, 27, 29]. Our combined immunohistochemical and molecular biological data reveal opposite results and show that in early (Clark I to Clark III) MM, ETB receptor expression is most intense in the radial growth phase, whereas in advanced MM, this expression predominates in the vertical growth phase. No down-regulation of ETB receptors in metastatic melanomas was evident by means of immunohistochemistry or RT-PCR. These discrepant findings may be explained by the limited number of cell lines analyzed previously [7, 17] and by the use of biopsy material in the present study. Indeed, melanoma cells in vitro may behave differently from those in patient samples and, as a consequence, alter their gene expression. Lahav et al. (1999) reported an ETB receptor antagonist that inhibits growth and induces cell death in human melanoma cells in vivo and in vitro [19]. This finding also favors a mitogenic and/or anti-apoptotic role of ETB receptors in MM.

In conclusion, whereas the exact role of ETs and their receptors in melanocytic neoplasms still remains largely unknown, we can conclude from our data that, in contrast to previous studies, the level of ETB receptor ex-

pression increases with successive tumor progression phases. These data strongly suggest that binding of ligand(s) to their ETB receptors induces a mitogenic or an anti-apoptotic effect in melanoma cells. Further studies are needed to unravel the role of ETB receptors in the signal transduction of MM tumor progression.

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References

1. Ahn GY, Butt KI, Jindo T, Yaguchi H, Tsuboi R, Ogawa H (1998) The expression of endothelin-1 and its binding sites in mouse skin increased after ultraviolet B irradiation or local injection of tumor necrosis factor α. J Dermatol 25:78–84
2. Alanen K, Deng DX, Chakrabarti S (2000) Augmented expression of endothelin-1, endothelin-3 and the endothelin-B receptor in breast carcinoma. Histopathology 36:161–167
3. Battistini B, Chailler P, DíOrleans-Juste P, Briere N, Sirois P (1993) Growth regulatory properties of endothelins. Peptides 14:385–399
4. Bieche I, Onody P, Laurendeau I, Olivi M, Vivaud D, Lidereau R, Vivaud M (1999) Real-time reverse transcription-PCR assay for future management of ERBB2-based clinical applications. Clin Chem 45:1148–1156
5. Ciotti P, Rainero ML, Nicolo G, Spina B, Garre E, Casabona F, Santi PL, Bianchi-Scarra G (1995) Cytokine expression in human primary and metastatic melanoma cells: analysis in fresh biopsy specimens. Melanoma Res 5:41–47
6. Clark WH, Elder DE, Guerry D, Braitman LE, Trock BJ, Schultz D, Synnestvedt M, Halpern AC (1989) Model for predicting survival in stage 1 melanoma based on tumor progression. J Natl Cancer Inst 81:1893–904
7. Eberle J, Weitmann S, Thieck O, Pech H, Orfanos CE.(1999) Downregulation of endothelin B receptor in human melanoma cell lines parallel to differentiation genes. J Invest Dermatol 112:925–932
8. Elder DE, Murphy GF (1990) Atlas of tumor pathology. Melanocytic tumors of the skin. Washington, Armed Forces Institute of Pathology, pp 154–161
9. Elshourbagy NA, Korman DR, Wu HL(1999) Molecular characterization and regulation of the human endothelin receptors. J Biol Chem 268:3873–3879
10. Greene MH. The genetics of hereditary melanoma and nevi. Cancer 86:2464–2477
11. Hara M, Yaar M, Gilchrest BA (1995) Endothelin-1 of keratinocyte origin is a mediator of melanocyte dendricity. J Invest Dermatol 105:744–748
12. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994
13. Hiraki H, Hoshi N, Hasegawa H,Tanigawa T, Emura I, Seito T, Yamaki T, Fukuda T, Watanabe K, Suzuki T (1997) Regular immunohistochemical localization of endothelin-1 and endothelin-B receptor in normal, hyperplastic and neoplastic human adrenocortical cells. Pathol Int 47:117–125
14. Imokawa G, Yada Y, Miyagishi M (1992) Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. J Biol Chem 267:24675–24680
15. Imokawa G, Miyagishi M, Yada Y (1995) Endothelin-1 as a new melanogen: coordinated expression of its gene and the tyrosinase gene in UVB-exposed human epidermis. J Invest Dermatol 105:32–37
16. Imokawa G, Yada Y, Kimura M, Morisaki N (1996) Granulocyte/macrophage colony-stimulating factor is an intrinsic keratinocyte-derived growth factor for human melanocytes in UVA-induced melanosis. Biochem J 313:625–631
17. Kikuchi K, Nakagawa H, Kadono T, Takafumi E, Byers HR, Mihm MC, Tamaki K (1996) Decreased ET(B) receptor expression in human metastatic melanoma cells. Biochem Biophys Res Commun 219:734–739
18. Kusafuka T, Wang Y, Puri P (1997) Mutation analysis of the RET, the endothelin-B receptor, and the endothelin-3 genes in sporadic cases of Hischsprung's disease. J Pediatric Surg 32: 501–504
19. Lahav R, Heffner G, Patternson PH.(1999) An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo. Proc Natl Acad Sci U S A 96:11496–11500
20. Masaki T (1989) The discovery, the present state, and the future prospects of endothelin. J Cardiovasc Pharmacol 5 [Suppl]:1–4
21. Moretti S, Pinzi C, Spallanzani A, Berti E, Chiarugi A, Mazzoli S, Fabiani M, Vallecchi C, Herlyn M (1999) Immunohistochemical evidence of cytokine networks during progression of human melanocytic lesions. Int J Cancer 84:160–168
22. Nelson JB, Chan-Tack K, Hedican SP, Magnuson SR, Opgenorth TJ, Bova GS, Simons JW (1996) Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. Cancer Res 56:663–668
23. Okazawa M, Shiraki T, Ninomiya H, Kobayashi S, Masaki T (1998) Endothelin-induced apoptosis of A375 human melanoma cells. J Biol Chem 273:12584–12592
24. Opdecamp K, Kos L, Arnheiter H, Pavan WJ (1998) Endothelin signalling in the development of neural crest-derived melanocytes. Biochem Cell Biol 76:193–199
25. Reid K, Turnley AM, Maxwell GD, Kurihara Y, Kurihara H, Bartlett PF, Murphy M. (1996) Multiple roles for endothelin in melanocyte development. Regulation of progenitor number and stimulation of differentiation. Development 122: 3911–3919
26. Rubanyi GM, Parker Bolelho LH (1991) Endothelins. Faseb J 5:2713–2720
27. Shichiri M, Kato H, Marumo F, Hirata Y (1997) Endothelin-1 as an autocrine/paracrine apoptosis survival factor for endothelial cells. Hypertension 30:1198–1203
28. Tsuboi R, Sato C, Oshita Y, Hama H, Sakurai T, Goto K, Ogawa H (1995) Ultraviolet B irradiation increases endothelin-1 and endothelin receptor expression in cultured human keratinocytes. FEBS Lett 371:188–190
29. Wu-Wong JR, Chiou WJ, Wang J (2000) Extracellular signal-regulated kinases are involved in the antiapoptotic effect of endothelin-1. J Pharmacol Exp Ther 293:514–521
30. Yada Y, Higuchi K, Imokawa G (1991) Effects of endothelins on signal transduction and proliferation in human melanocytes. J Biol Chem 266:18352–18357
31. Yanagisawa M, Kurihara H, Kimur S, et al. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411–415
32. Yohn JJ, Smith C, Stevens T, Hoffman TA, Morelli JG, Hurt DL, Yanagisawa M, Kane MA, Zamora MR (1994) Human melanoma cells express functional endothelin-1 receptors. Biochem Biophys Res Commun 201:449–457
33. Yoshida H, Kunisada T, Kusakabe M, Nishikawa S, Nishikawa SI (1996) Distinct stages of melanocyte differentiation revealed by analysis of nonuniform pigmentation patterns. Development 122:1207–1214