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Expression of Endothelins and Their Receptors in Nonmelanoma Skin Cancers

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Background: Endothelins are paracrine peptides with growth-promoting and vasoactive functions for a variety of cell types. Elevated activation of the endothelin signaling pathway induces cell proliferation and/or survival and is implicated in a variety of malignancies. Increased endothelin 1 was described in solar lentigines in previous reports, raising the possibility that the endothelin pathway may be of significance in keratinocyte proliferation-related disorders. However, detailed investigation on endothelins in skin malignancies is lacking.

Objectives: This study aims to survey the expression of endothelins and their receptors in keratinocyte-derived benign and malignant tumors of the skin and to test the effects of endothelin inhibitors on the growth and survival of cultured keratinocytes.

Methods: Quantitative polymerase chain reaction was used to measure the level of gene transcription of three endothelins (ET-1, -2, and -3) and two endothelin receptors (ETRA and ETRB). The genes with significant messenger ribonucleic acid (mRNA) expression abnormalities were confirmed with immunohistochemical analysis to examine expression differences at the protein levels. To analyze the effect of endothelin inhibitors on the keratinocyte growth and survival, keratinocytes were cultured in the presence of various concentrations of endothelin inhibitors and subjected to tetrazolium bromide assay to quantify the cell numbers over time.

Results: ET-1 mRNA was found to be significantly up-regulated in seborrheic keratosis and basal cell carcinoma. However, no significant expression increase was found in actinic keratosis, Bowen's disease, or squamous cell carcinoma. Immunohistochemical analysis of ET-1 peptide confirmed increased expression. In cultured keratinocytes, peptide inhibitors of the endothelin pathway resulted in a marked reduction in cell survival.

Conclusion: The endothelin signaling pathway, especially ET-1, is activated in basoloid keratinocyte neoplasms of the skin, such as basal cell carcinoma and seborrheic keratosis. Blockade of this pathway can reduce cell survival in vitro. Therefore, endothelin inhibitors potentially offer a novel method for the treatment of some keratinocyte-derived skin tumors.

Antécédents: Les endothélines sont des peptides à action paracrine ayant des fonctions anabolisantes et vasoactives dans plusieurs types de cellules. Une plus forte activation des voies de signalisation de l'endothéline produit une prolifération ou une survie des cellules et se trouve associée à un grand nombre de malignités. Une augmentation de l'endothéline 1 a été rapportée dans des cas antérieurs de lentigo sénile, soulevant la possibilité que les voies de signalisation de l'endothéline pourraient jouer un rôle important dans les troubles liés à la prolifération de kératinocytes. Toutefois, il n'y a pas assez d'enquêtes sur le rôle des endothélines dans les cancers de la peau.

Objectifs: La présente étude vise à explorer l'expression des endothélines et de leurs récepteurs dans les tumeurs cutanées bénignes ou malignes dérivées des kératinocytes et à tester les effets des inhibiteurs des endothélines sur la survie et la croissance des kératinocytes en laboratoire.

Méthodes: Une réaction en chaîne de la polymérase quantitative a été utilisée pour mesurer le niveau de transcription des gènes de trois endothélines (ET-1, -2, et -3) et de deux récepteurs de l'endothéline (ETRA et ETRB). Les gènes présentant des anomalies considérables dans l'expression de l'ARN messager ont été confirmés par une analyse de l'immunohistochimie pour examiner les différences dans l'expression au niveau des protéines. Afin d'analyser les effets des inhibiteurs de l'endothéline sur la croissance et la

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survie des kératinocytes, on a recouru à des cultures de kératinocytes en présence de diverses concentrations d'inhibiteurs de l'endothéline exposées à des doses de tétrazolium bromure afin de quantifier le nombre de cellules au fil du temps.

Résultats: Une régulation positive a été constatée dans l'ARN messager de l'endothéline 1 des verrues séborrhéiques et des carcinomes basocellulaires. Toutefois, aucune hausse dans l'expression n'a été constatée au niveau des kératoses actiniques, de la maladie de Bowen, ou des carcinomes squameux. L'analyse de l'immunohistochimie des peptides endothéliales 1 a confirmé une augmentation de l'expression. Dans les cultures de kératinocytes, les inhibiteurs de peptides des voies de l'endothéline ont mené à une baisse marquée dans la survie des cellules.

Conclusion: La voie de signalisation de l'endothéline, surtout l'endothéline 1, est activée dans les tumeurs kératinocytes basaloïdes de la peau, telles que les carcinomes cellulaires et les verrues séborrhéiques. Le blocage de cette voie peut réduire le temps de survie de la cellule *in vitro*. Par conséquent, les inhibiteurs de l'endothéline offrent une nouvelle méthode possible du traitement de certaines tumeurs cutanées produites par les kératinocytes.

ENDOTHELINS (ETs) belong to a family of 21-amino acid peptides comprising three isoforms in human cells. Originally, ET was identified as an endothelium-derived vasoconstrictor that is cleaved and released by the ET-converting enzyme.¹ ETs function through two subtypes of guanine nucleotide-binding regulatory protein (G protein)-coupled receptors, termed ETRA and ETRB, respectively. At physiologic concentrations, ET-1 and ET-2, but not ET-3, bind to ETRA, whereas all three ET ligands bind to ETRB with a similar affinity.² In addition to vasoconstrictor activity, the ET system plays important roles in cell proliferation, cell adhesion, migration, and angiogenesis through both autocrine and paracrine pathways.³ For example, it has been shown that ET can promote angiogenesis by increasing vascular endothelial growth factor production in a hypoxia-inducible factor α -dependent manner.⁴ The ET system has been implicated in a variety of cancers, including carcinomas of the prostate, ovary, colon, cervix, breast, kidney, lung, colon, and central nervous system, as well as melanoma,⁵ Kaposi's sarcoma, and bone metastasis.⁶ Several inhibitors have been developed to interfere with the function of the ET system, and some have been approved for treating some cancers and other diseases.⁷

Nonmelanoma skin cancer (NMSC) is the most common cancer affecting human beings.^{8,9} It causes considerable morbidity and can cause mortality. There are many types of NMSC. Most of them are derived from keratinocytes, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), which account for 75% and 20% of all skin cancers, respectively. A precursor of SCC is actinic keratosis (AK), also known as solar keratosis. AK, Bowen's disease (BD), and SCC are considered to represent a spectrum of cutaneous squamous cell neoplasms. BD, or SCC *in situ*, can progress to become invasive SCC. Seborrheic keratoses (SK) are benign, flat, raised, or pedunculated lesions, histologically

described as basal cell papillomas; they have no malignant potential. Although considered invasive carcinoma, BCC is not prone to metastasize to distant regions of the body. Because of their morphologic resemblance to keratinocytes at the basal layers of the epidermis, both SK and BCCs are considered basaloid neoplasms compared with AK, BD, and SCC, which show more similarities to squamated, suprabasal keratinocytes in the epidermis. Common therapeutic modalities for NMSC include surgery, topical chemotherapy, and radiotherapy.¹⁰

Many environmental factors, including ultraviolet (UV) and ionizing radiation, contribute significantly to skin cancer development.¹¹ The most frequently occurring molecular event in NMSC development is *p53* mutation.^{12,13} More recently, dysregulation of the sonic hedgehog pathway has been intensively studied in NMSC development, especially BCC.¹⁴ Since skin keratinocytes produce ETs and express the cognate receptors,¹⁵ the ET-ETR autocrine growth stimulation pathway might be altered in uncontrolled cell growth in keratinocyte-derived neoplasms. However, to date, very little is known about the ET pathway in malignant keratinocytic neoplasms. Previously, ET-1 has been observed in pigmented keratinocyte lesions, such as solar lentigo and SK.¹⁶ Given that these disorders have both increased keratinocyte number and can demonstrate increased melanin pigmentation, it is not clear if ET-1 is implicated in pigmentation only or pigmentation and keratinocyte proliferation or survival. It has also been reported that ET-1 and ET-2 levels are increased in the plasma of patients in AK and BCC, although the exact cellular source was not reported.¹⁷ In this study, we examined the messenger ribonucleic acid (mRNA) levels of the ET system in five keratinocyte-derived proliferative conditions, including both pigmented and nonpigmented lesions, and determined the effects of ETR antagonists on keratinocyte cell survival. Our findings revealed increased ET pathway activation in basaloid neoplasms (SK and BCC)

independent of pigmentation status, suggesting a role in promoting keratinocyte tumorigenesis. This was supported by the demonstration that ET antagonists caused the death of cultured keratinocytes in vitro.

Materials and Methods

Patient Recruitment and Tissue Sample Collection

The study was approved by the University of British Columbia Clinical Ethics Board. Patients with a histologic diagnosis of BCCs, BD, and SCCs in the Dermatological Surgery Unit of the Department of Dermatology and Skin Science were recruited. Approximately 3 mm-sized samples were obtained from the center of the biopsy-confirmed skin cancers prior to Mohs' micrographic surgery debulking and were bisected. One portion was fixed in 10% formalin and used for histologic confirmation (by Dr. M. Martinka, Department of Pathology and Laboratory Medicine), whereas the other part was placed in RNAlater solution and stored at 4°C for short-term storage and to -80°C freezer until ribonucleic acid (RNA) extraction. Clinically evident SK and AK from the face and neck region of the patients were also biopsied in a similar fashion, with half from each sample used for histologic confirmation and the other half used for RNA extraction. As normal tissue controls, the excessive normal skin tissue left over from post-Mohs' wound repair was collected from each patient. They are subjected to the same histologic confirmation and RNA extraction (see below). This study analyzed clinical samples from 48 patients, including 10 with nodular BCCs, 12 with SK, 8 with BD, 5 with AK, and 13 with invasive SCCs.

Quantification of mRNA of Genes in the ET Signaling Pathway

RNA preparation and quantitative reverse-transcription polymerase chain reaction (PCR) were conducted as previously described.⁵ Briefly, total RNA was extracted from the specimens by homogenization in Trizol solution (Invitrogen, Burlington, ON) and purified using Mini-Elute Cleanup Kit (Qiagen, Mississauga, ON). One microgram of total RNA was used for complementary deoxyribonucleic acid (cDNA) synthesis with random hexamers using the SuperScript II First-Strand Synthesis System (Invitrogen). The cDNA reaction was diluted, and 1/250 of the whole cDNA mixture (equivalent to 4 ng of total RNA) was used as the template for each quantitative PCR. The primers were designed using Applied Biosystems Inc's (ABI, Foster City, CA) *Primer Express* software and are listed in Table 1.

The PCRs were performed with a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Alameda, CA) using ABI's master mix. Each PCR was performed in quadruplicate. The amplification conditions were 95°C for 10 minutes followed by 42 cycles of 95°C for 15 seconds and 60°C for 1 minute. The cycle number of threshold (CT) was recorded for each reaction. The CT value of each target gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal ribonucleic acid (rRNA). The data are normalized as copies of target gene mRNA per 1 million copies of GAPDH mRNA or 18S rRNA in the same sample using the formula $10^{6/2\Delta CT}$ (where $\Delta CT = CT_{\text{target gene}} - CT_{\text{GAPDH or 18S rRNA}}$).

Probability values were calculated by using the two-tailed Student's *t*-test.

Table 1. Primers Used for Quantitative Polymerase Chain Reaction

Genes	5'-Primer Sequence	3'-Primer Sequence	Amplicon Length (bp)
GAPDH	AAGATCATCAGCAATGCCTCC	TGGACTGTGGTCATGAGTCCTT	104
ET-1	ACAAACCAGGTCGGAGACCAT	CCGAAGGTCTGTCACCAATGT	126
ET-2	AGTCCCCTGCAGACGTGTTT	ACTGTGGAATGTCCCTCAGC	85
ET-3	TGGATCAACACTCCCGAACAG	TCATATCTCCCCACACAAGCG	149
ETRA	AGCGTCGAGAAGTGGCAAAA	ACATCGGTTCTTGTCATTTCG	125
ETRB	TTGCTTCATCCCGTTCAGAA	TTCCCGTCTCTGCTTTAGGTG	195
18S rRNA	GTAACCCGTTGAACCCCAT	CCAT CCAATCGGTAGTAGCG	151

ET = endothelin; ETR = endothelin receptors; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Immunohistochemistry

For immunohistochemical assessment of ET-1 expression, 4 μ m paraffin-embedded sections were stained with mouse immunoglobulin G1 monoclonal antibody against human ET-1 at 1:500 dilutions (clone TR.ET.48.5, Affinity Bioreagents, Burlington, ON). Antigen retrieval was by microwaving the slide section for 4 minutes as described previously.^{5,18,19} This antibody was shown to be specific to ET-1 and had been used for immunohistochemical detection of human ET-1 on paraffin-embedded biopsies by others.²⁰

The staining signals were visualized using DAKO's LSAB[®]+System and Liquid DAB Substrate-Chromogen System (DAKO Corporation, Carpinteria, CA). Substitution of normal mouse serum for the primary antibody served as the negative control.

Cell Culture, ET Inhibitor Treatment, and Keratinocyte Proliferation Assay

Human HaCat cells, which were immortalized keratinocytes derived from normal keratinocytes of non-sun-exposed skin on the back of a man,²¹ were cultured in 96-well plates in 5% fetal bovine serum Dulbecco's Modified Eagle Medium (DMEM). Cells were incubated in serum-free DMEM for 24 hours. Then 100 nM of ETRA antagonist BQ123, ETRB antagonist BQ788, or both (Sigma, St. Louis, MO) in serum-free DMEM was added to the culture. The medium was changed every 3 days. To assay for cell survival, 2,5-diphenyltetrazolium bromide (MTT) was added to the cell cultures on day 0 (after 24 hours of a serum-free washout period), day 3, and day 6, as previously described.⁵ Each test was performed in

quadruplicate. Student's *t*-test was performed to determine the level of significance at each time point using day 0 data as the reference. This experiment was performed three times.

Results

mRNA Expression of ETs and Their Receptors in NMSCs

Among the five genes of the ET signaling pathway (ET-1, ET-2, ET-3, ETRA, and ETRB), four (ET-1, ET-2, ETRA, and ETRB) were readily detectable among the skin biopsies analyzed further (Table 2). In contrast, since ET-3 was not expressed at detectable levels, it was excluded from further analysis and discussion.

In BCCs, there was a strong increase in expression of ET-1, ET-2, and ETRB compared with in normal skin, although expression of ETRA was not altered significantly (Figure 1). In SK, significant overexpression of ET-1 was also observed compared with in normal skin, although the level of transcriptional increase is much less than observed in BCC samples (Figure 2). However, there was no significant expression increase in the other genes analyzed (see Figure 2). Unlike BCC and SK, AK, BD, and SCC showed no significant expression differences in the genes analyzed (see Table 2).

Increased ET-1 Protein Expression in BCC and SK Biopsies

Given that ET-1 mRNA was consistently overexpressed in both SK and BCC lesions (see Figures 1 and 2), we further

Table 2. Expression Survey of Transcription of Genes Involved in the Endothelin Pathway in Keratinocyte-Derived Neoplasms of the Skin*

Type of Nonmelanoma Skin Tumor	ET-1		ET-2		ETRA		ETRB	
	L/N Ratio	p** Value	L/N Ratio	p Value	L/N Ratio	p Value	L/N Ratio	p Value
BCC (n = 10)	46.4	.0029	23.4	.0262	1.4	NS	38.5	.0070
SK (n = 11)	3.6	.0075	0.2	.00085	0.91	NS	2.1	NS
SCC (n = 13)	2.2	NS	2.3	NS	0.7	NS	1.6	NS
AK (n = 5)	3.7	NS	0.1	NS	0.9	NS	1.3	NS
BD (n = 8)	0.6	NS	1.4	NS	0.1	NS	0.3	NS

AK = actinic keratosis; BCC = basal cell carcinoma; BD = squamous cell carcinoma in situ (SCC in situ or Bowen's disease); ET = endothelin; ETR = endothelin receptor; NS = not significant; SCC = squamous cell carcinoma; SK = seborrheic keratosis.

*The quantitative polymerase chain reaction (PCR) analysis was performed as described in the Materials and Methods. L/N ratios: For each gene in a sample that was analyzed by quantitative polymerase chain reaction, the concentration of the messenger ribonucleic acid (mRNA) (copies per million copies of glyceraldehyde-3-phosphate dehydrogenase mRNA in the same sample) in the skin lesion was divided by the mRNA concentration of the control normal skin biopsy of the same patient.

**p values represent the results of Student's *t*-test, two-tailed analysis. NS: *p* > .05.

Figure 1. Endothelin (ET) and endothelin receptor (ETR) messenger ribonucleic acid (mRNA) expression in basal cell carcinoma. The expression of ET-1, ET-2, ETRA and ETRB from control and lesion samples of 10 patients was analyzed. The mRNA expression of each individual endothelin gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A) or 18S ribosomal ribonucleic acid (rRNA) (B) of the same sample, and shown as copies of mRNA per million copies of GAPDH mRNA or 18S rRNA.

examined its protein expression and cellular localization using immunohistochemistry. As shown in Figure 3, a small fraction of keratinocytes (less than 20%) in normal skin were stained positive for ET-1 expression, and these cells were restricted to the basal layer (Figure 3B). In contrast, strong ET-1 staining was present in the vast majority of keratinocytes (more than 90%) of both BCC and SK lesions (Figure 3, D and F). The expression was present in cells throughout the lesions, not just restricted in the basal layer.

Effect of ETR Blockade on Keratinocyte Growth in Culture

To determine the functional impact of an activated ET system on keratinocytes, immortalized keratinocytes (HaCaT cell line) were treated with specific ETR antagonists, BQ123 (ETRA inhibitor) or BQ778 (ETRB inhibitor), which blocks the function of endothelins, including ET-1. As shown in Figure 4, the endothelin blockade significantly reduced the cell number of cultured keratinocytes ($p < .01$, two-tailed *t*-test).

Figure 2. Endothelin (ET) and endothelin receptor (ETR) messenger ribonucleic acid (mRNA) expression in seborrheic keratosis. The expression of ET-1, ET-2, ETRA, and ETRB from control and lesion samples of 12 patients was analyzed. The mRNA expression of each individual ET gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A) or 18S ribosomal ribonucleic acid (rRNA) (B) of the same sample and shown as copies of mRNA per million copies of GAPDH mRNA or 18S rRNA.

Figure 3. Immunohistochemical detection of endothelin 1 (ET-1) protein expression in seborrheic keratosis (SK) and basal cell carcinoma (BCC). Paraffin-embedded sections of normal skin (A, B), SK (C, D), and BCC (E, F) were stained with monoclonal anti-ET-1 antibody (B, D, F) or normal mouse serum (A, C, E), as described in the Materials and Methods.

Discussion

In this study, we report gene expression changes in the ET signal transduction pathway in NMSCs using the house-keeping genes GAPDH and rRNA as the references.

Although it is true that GAPDH gene expression is rarely static during cell proliferation and differentiation, the magnitude of expression difference (generally less than twofold) is usually not on the scale of ET gene activation observed in this study.^{22,23} Therefore, the observation of transcriptional activation of ET pathway genes in BCC (more than 23-fold) and SK (3.6-fold) is unlikely to be due to the biased reference gene used for the study. Further, immunohistochemistry (which is not affected by RNA quantification of the GAPDH gene) of ET-1 on normal skin, SK, and BCC verified increased expression of ET-1 expression in the SK and BCC samples. Finally, when using 18S rRNA as the reference gene, which was less variable than the GAPDH gene,²² the conclusions did not change (see Figures 1 and 2).

Based on the cell morphology, the keratinocyte-derived skin cancers can be classified into two categories: basaloid keratinocytic neoplasms and squamous cell neoplasms. The cell morphology of the basaloid keratinocytic neoplasms (such as SK and BCC) resembles the proliferating keratinocytes in the basal layer of the epidermis or the hair follicles. In contrast, the cells of the squamous cell neoplasms (AK, BD, and SCC) are similar to the differentiating keratinocytes in the upper layer of the epidermis. It is interesting that ET pathway genes, especially ET-1, showed evidence of transcriptional activation only in basaloid keratinocyte neoplasms.

Previously, Teraki and colleagues also reported ET-1 immunostaining in SK cells and in normal epidermal keratinocytes of the basal layer.¹⁶ Therefore, our result further confirmed this observation in SK. The observation of increased expression ET-1, ET-2, and ETRB by BCC is reported here for the first time.

Teraki and colleagues speculated that increased expression of ET-1 in SK was possibly contributing to increased

Figure 4. Effects of endothelin antagonists on keratinocyte survival in vitro. Immortalized human keratinocytes (HaCaT) were treated with 100 nM of endothelin receptor (ETRA) antagonist (BQ123), ETRB antagonist (BQ788), or both. The number of cells in each culture compartment was determined by tetrazolium bromide at different time points. Using 100% for the control group at day 0, the percentage of treated groups at day 3 and day 6 was plotted ($p < .01$, Student's *t*-test). Each assay was performed in triplicate, with the error bars representing standard deviations for each data point.

pigmentation.¹⁶ However, since only about half of the SK and none of the BCCs analyzed in this investigation were pigmented, increased expression of ET pathway genes is not likely to be of major significance in regulating lesional pigmentation. The observation that ET pathway blockade resulted in marked decrease in keratinocyte survival in serum-free medium (see Figure 4) suggests another possible function of activated ET pathways in nonmelanoma skin cancers. That is, it potentially increases cell survival.

A major etiologic factor for NMSC development is excessive exposure to UV radiation as SCC and BCC occur primarily on sun-exposed areas of the skin.²⁴ ET production is stimulated by a variety of cytokines, including interleukin (IL)-1 α , tumor necrosis factor (TNF)- α , and platelet-derived growth factor.^{15,25,26} UV irradiation can induce IL-1 α and TNF- α , which can result in overexpression of ET-1.^{27,28} Our study suggests that ET-1 may be an important mediator for UV-mediated carcinogenesis.

Several ET and ETR pathway inhibitors have been developed to interfere with the function of the ET pathway in tumor cells. In addition, some ET inhibitors are approved or investigated for treating cancers and other diseases.^{6,29} The results reported in this study raise the possibility of ET inhibitors being used as a therapy for SK and BCC in the future.

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