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Endothelin-3 production by human rhabdomyosarcoma: A possible new marker with a paracrine role

Arianna Palladini[a], Annalisa Astolfi[a], Stefania Croci[a], Carla De Giovanni[a,b], Giordano Nicoletti[c], Angelo Rosolen[d], Francesca Sartori[d], Pier-Luigi Lollini[a,b], Lorena Landuzzi[c,], Patrizia Nanni[a,b]*

[a]*Cancer Research Section, Department of Experimental Pathology, University of Bologna, Viale Filopanti 22, I-40126 Bologna, Italy*
[b]*Interdepartment Center for Cancer Research "G. Prodi", University of Bologna, Bologna, Italy*
[c]*Istituti Ortopedici Rizzoli, Via di Barbiano 1/10, I-40136 Bologna, Italy*
[d]*Clinic of Paediatric Haematology Oncology, Department of Paediatrics, University of Padova, Via Giustiniani 3, I-35128 Padova, Italy*

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ABSTRACT

Several autocrine and paracrine growth factor circuits have been found in human rhabdomyosarcoma cells. In this study we show that endothelin-3 (ET-3), a vasoactive peptide, is produced by human rhabdomyosarcoma cell lines, whereas it is not expressed by human sarcoma cell lines of non-muscle origin. We did not find evidence of a significant autocrine loop; nevertheless ET-3 produced by rhabdomyosarcoma cells can act as a paracrine factor, since it promotes migration of endothelial cells. Moreover ET-3 is present in plasma of mice bearing xenografts of human rhabdomyosarcoma cells, and may be potential new marker of the human rhabdomyosarcoma to be studied further.

1. Introduction

Rhabdomyosarcoma is the most common soft-tissue sarcoma in children; it arises as a consequence of regulatory disruption of the growth and differentiation of skeletal muscle progenitor cells.[1] The unrestricted proliferation and growth of rhabdomyosarcoma cells is sustained by multiple autocrine and paracrine growth factor circuits.[2–8]

Endothelin (ET) family comprises three 21-aminoacid peptides: ET-1, ET-2 and ET-3. ET-1 and ET-2 have similar structures, whereas ET-3 differs from ET-1 structure in 6 out of 21 positions[9]. ET-3 is expressed mainly in the brain; this peptide is produced also in kidney and by gastrointestinal stromal and lung epithelial cells.[10] Endothelin receptors are two G-protein coupled receptors. Endothelin A receptor (ET_AR) is specific for ET-1, whereas endothelin B receptor (ET_BR) exhibits similar affinities for all the three isopeptides.[11]

The expression of ET-1 and ET_AR has been identified in many human cancer cell lines and tumours, including prostate, ovarian, lung, colon, cervical carcinomas and glioma. In many of these tumours ET-1 acts through paracrine mechanisms promoting the growth of local stromal tissue.[10,12] ET-1 is involved in various stages of neovascularization from endothelial cell proliferation to stimulation of endothelial cell migration, invasion, protease production and tube formation.[13,14] ET-3 is also involved in the angiogenic process: this peptide regulates the production of vascular endothelial growth factor.[13,15,16] The role of ET-3 in human tumours has

* *Corresponding author:* Tel.: +39 051 2099399; fax: +39 051 242169.
E-mail address: landuzzi@cancer.unibo.it (L. Landuzzi).
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not been studied in depth. The expression of ET-3 and ET_BR increases in human breast carcinomas[17] but is reduced in Ewing's sarcoma.[18] Moreover ET-3, along with ET-1, regulates local and metastatic growth of melanoma through the binding to endothelin B receptor.[19] ET-3 acts as a paracrine growth factor on the stromal compartment population in ovarian cancer.[20] Finally both ET-3 and ET-1 increase the proliferation, migration and invasiveness of Kaposi's sarcoma cells.[21,22]

In this study we have investigated the presence of autocrine or paracrine endothelin-3 circuits in human rhabdomyosarcoma. We found that ET-3 is produced by human rhabdomyosarcoma cell lines, whereas it is not expressed by human sarcoma cell lines of non-muscle origin. We also show here that ET-3 produced by rhabdomyosarcoma cells can play a role as a paracrine factor on endothelial cells.

2. Materials and methods

2.1. Cell lines and culture conditions

A panel of 24 cell lines from human sarcomas (rhabdomyosarcoma, osteosarcoma and Ewing's sarcoma) was used (Table 1). Rhabdomyosarcoma cells were routinely cultured in Dulbecco's minimal essential medium (DMEM) with the addition of 10% (v/v) heat-inactivated fetal bovine serum (FBS), osteosarcomas and Ewing's sarcomas were cultured in Iscove's modified Dulbecco's medium + 10% FBS. All cell lines were maintained in a 7% CO_2 humidified atmosphere at 37 °C. Culture media were purchased from Invitrogen (Milan, Italy). Human umbilical vein endothelial cells (HUVEC, purchased from Clonetics, Bio-Whittaker, Cambrex, Milano) were cultured in EGM-2 medium (Clonetics) in a 5% CO_2 humidified atmosphere at 37 °C.

To induce cell differentiation, rhabdomyosarcoma cells (clones RD/12 and RD/18) were seeded (8000 cells/cm^2) in complete medium and shifted on day 1 to differentiation medium DMEM + 2% (v/v) horse serum. At different time points, cells were harvested and counted and supernatants collected for ET-3 determination.

2.2. Functional studies

The human rhabdomyosarcoma cell lines RD/18, RD/12, RMZ-RC2 and the ovarian carcinoma cell line SKOV3 (kind gift of Dr. Serenella Pupa, Istituto Nazionale Tumori, Milan, Italy) were seeded in 24-well plates (RMZ-RC2 100×10^3 cells/well, RD/12 20×10^3cells/well, RD/18 and SKOV3 40×10^3 cells/well) in DMEM + 10% FBS for rhabdomyosarcoma cell lines, in Roswell Park Memorial Institute medium (RPMI) + 10% FBS for SKOV3. The following day cells were shifted to DMEM + 2% horse serum (rhabdomyosarcoma cell lines) or RPMI + 1% (v/v) FBS (SKOV3) supplemented with 1–1000 nM recombinant human ET-1 (r-hET1) (Bachem, Bubendorf, Switzerland). Cells were harvested and counted after 72 h of treatment.

The effects of cell pretreatment with antagonists of endothelin receptors were tested on RMZ-RC2 rhabdomyosarcoma cells. The day after seeding cells were treated with BQ123 (Bachem), ET_AR antagonist,[23] or BQ788 (Bachem) ET_BR antagonist,[24] both at 100 nM concentration. Cells were incubated for 20 min at 37 °C. Then r-hET1 or r-hET3 (Phoenix Pharmaceuticals, Belmont, CA, USA) at 10 nM final concentration were added to cells in the presence of the receptor antagonists. Experimental controls included cells treated with each

Table 1 – Human sarcoma cell lines used throughout the study

Cell line	Histological type	Origin and/or reference
RD/18	Embryonal rhabdomyosarcoma (clone of RD cell line)	[33]
RD/12	Embryonal rhabdomyosarcoma (clone of RD cell line)	[33]
CCA	Embryonal rhabdomyosarcoma	[34,35]
RMZ-RC2	Alveolar rhabdomyosarcoma	[35,36]
SJ-RH30	Alveolar rhabdomyosarcoma	Dr. P. Houghton (St. Jude Children's Hospital, Memphis, TN)
SJ-RH4	Alveolar rhabdomyosarcoma	Dr. P. Houghton (St. Jude Children's Hospital, Memphis, TN)
U-2 OS	Osteosarcoma	ATCC (Rockville, MD)
Saos-2	Osteosarcoma	ATCC (Rockville, MD)
MOS	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[35]
SARG	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[37]
MG-63	Osteosarcoma	ATCC (Rockville, MD)
IOR/OS7	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[35]
IOR/OS9	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[35]
IOR/OS10	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[35]
IOR/OS14	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[35]
IOR/OS15	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[35]
IOR/OS18	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[35]
IOR/OS20	Osteosarcoma	Istituti Ortopedici Rizzoli, Bologna, Italy[35]
6647	Ewing's sarcoma	Dr. T.J. Triche (Children's Hospital, Los Angeles, CA)
TC-71	Ewing's sarcoma	Dr. T.J. Triche (Children's Hospital, Los Angeles, CA)
SK-ES	Ewing's sarcoma	ATCC (Rockville, MD)
SK-N-MC	Ewing's sarcoma (Askin's tumour)	ATCC (Rockville, MD)
RD-ES	Ewing's sarcoma	ATCC (Rockville, MD)
LAP-35	Ewing's sarcoma (PNET)	Istituti Ortopedici Rizzoli, Bologna, Italy[38]

single component alone (r-hET-1, r-hET3, BQ123, BQ788). After 72 h of treatment, cells were harvested and counted.

2.3. Expression of endothelins

The expression of genes coding for ETs (*EDN1, EDN2* and *EDN3*) and endothelin receptors ET_AR (*EDNRA*) and ET_BR (*EDNRB*) was investigated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cell lines and from surgical specimens of human skeletal muscle and rhabdomyosarcomas by TRIzol reagent; 1 μg of total RNA was reverse-transcribed using reverse transcriptase in the presence of oligo-dT (cell lines) or random hexamers (surgical specimens). cDNA was amplified with specific primer pairs for ET-1, ET-2, ET-3, ET_AR and ET_BR genes (Table 2) and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Clontech, Palo Alto, CA). The amplification of GAPDH was performed to check that comparable amounts of cDNA were analysed. All reagents were purchased from Invitrogen. The amplification products (452 bp for GAPDH, 582 bp for ET-1, 197 bp for ET-2, 493 bp for ET-3, 366 bp for ET_AR, 530 bp for ET_BR) were visualized on ethidium bromide stained agarose gels.

ET-1 and ET-3 expression was also analysed by quantitative real-time PCR using an ABI Prism 5700 sequence detection system (Applied Biosystems, Applera, Milan, Italy). Real-time PCR was performed using SYBR® Green PCR Master Mix Reagents (Applied Biosystems). ET-1-CDSa and ET-3-CDS primer pairs, designed using Primer Express software version 2.0, and ET-1-CDSb and ET-3-UTR primer pairs, reported in literature, were synthesised by Invitrogen (Table 2). GAPDH was used as an endogenous reference gene. As the PCR primer pairs were found to have similar amplification efficiencies through serial dilutions of positive samples, relative quantification of the mRNA levels of ET-1 and ET-3 was determined using the ΔΔCt method. Briefly, the amount of target gene was normalized to the endogenous reference gene (GAPDH) and its expression in tumour cell lines was calculated relative to normal human skeletal muscle. The results are expressed as log-fold difference in expression of tumour cell lines relative to muscle.

2.4. Detection of soluble endothelins

2.4.1. ELISA

To detect ET-1 and ET-3 produced by cell lines, culture media were collected 48 h after seeding of 10^6 cells in 5 ml complete medium. Soluble peptide production was determined by ELISA test according to manufacturer's instructions (Assay Designs, Ann Arbor, MI, USA). The cross reactivity of ET-1 ELISA test is 3.3% for ET-2 and lower than 0.1% for ET-3; the cross reactivity of ET-3 ELISA test is 19.7% for ET-2 and 1.8% for ET-1, as reported by manufacturer's data sheets.

2.4.2. Western blot

The production of ET-3, which is a small peptide, was studied according to the method developed by Schagger.[25] Media conditioned by rhabdomyosarcoma cells were collected after 5 days of culture. Fifteen microliters of each supernatant was mixed with an equal amount of Tricine sample buffer (Bio-Rad, Milan, Italy) containing 10% (v/v) of beta-mercaptoethanol, denatured at 95 °C for 5 min and separated into a 16.5% (w/v) Tris–Tricine polyacrylamide gel (Ready Gel, Bio-Rad). The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for 30 min at 100 V, subsequently blocked with 5% (w/v) non-fat dry milk/PBS with 0.1% (v/v) Tween 20 for 1 h, and incubated overnight at 4 °C with an anti-hET-3 rabbit polyclonal antibody (US Biomax, Rockville, MD, USA) diluted 1:50 in 2% non-fat dry milk/PBS with 0.1% Tween 20. The presence of ET-3 was detected using a colorimetric reaction (Opti-4CN Substrate kit, Bio-Rad) after 2 h incubation with a horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.5. Migration assay

RD/18 cells were seeded (27,000 cells/cm^2) in DMEM + 10% FBS and shifted on day 1 to RPMI + 1% FBS. Supernatants were collected 72 h after seeding. Migration assay was performed using Transwell chambers with 8-μm pore size, polyvinylpyrrolidone-free polycarbonate filters (Corning, Cambridge, MA). Control medium (RPMI + 1% FBS) or RD/18 conditioned medium was put in the lower compartment while 10^5 HUVEC were

Table 2 – Primer pairs used throughout the study

Gene	Analysis	Sequence (5′–3′)	Length (bp)	Origin or reference
ET-3	Conventional PCR	For: TGTGAGTGTGGAGATGTTATCC Rev: TTCTCTCTGATACCATCTTGCC	493	[20]
ET_AR	Conventional PCR	For: CACTGGTTGGATGTGTAATC Rev: GGAGATCAATGACCACATAG	366	[20]
ET_BR	Conventional PCR	For: ACTGAATAGCCACCAATCTT Rev: ACTGAACAGCCACCAATCTT	530	[20]
ET-1-CDSa	Real-time PCR	For: GAGTGTGTCTACTTCTGCCACCTG Rev: TCCAAGGCTCTCTTGGACCTAG	102	Our laboratory
ET-1-CDSb	Real-time PCR	For: GGAAAAGACTGTTCCAAGC Rev: GGTTGTGGGTCACATAACG	193	[39]
ET-3-CDS	Real-time PCR	For: TGCCACCTGGACATCATTTG Rev: GGCCCCGCAGACCTCTT	101	Our laboratory
ET-3-UTR	Real-time PCR	For: CGAGCTTACTGTGAGTGTGGAGATG Rev: CCCACTCAAATGCCGTTTCC	116	[40]

For, forward; Rev, reverse

seeded in the upper compartment. To evaluate the specificity of endothelin B receptor-mediated effects, HUVEC were treated for 15 min at 37 °C before seeding with increasing doses of the BQ788 receptor antagonist. The chambers were incubated for 5 h at 37 °C in a 7% CO_2 humidified atmosphere. Cells that migrated through the filter reaching the lower chamber were counted at the inverted microscope.

2.6. *Mice and in vivo studies*

Athymic Crl:CD1-*Foxn1*^{nu} female mice (4–6 weeks old) were purchased from Charles River (Calco, Italy). All of the experiments with animals were in accord with institution guidelines and were approved by the institution committee for animal use and care. Rhabdomyosarcoma xenografts were induced in twelve animals by subcutaneous injection of 5×10^6 RD/18 cells in the leg. EDTA-treated blood samples were collected from 6 control and 12 tumour-bearing mice; plasma was obtained by centrifugation.

3. Results

The expression of *ET-3* gene was studied in six human rhabdomyosarcoma cell lines (two clonal derivatives of cell line RD -RD/12 and RD/18- and CCA of embryonal histotype; RMZ-RC2, SJ-RH30 and SJ-RH4 of alveolar histotype) and in normal skeletal muscle. All rhabdomyosarcoma cell lines strongly expressed *ET-3* gene, whereas normal skeletal muscle showed a very low expression (Fig. 1). RT-PCR analysis of a representative panel of human sarcoma cell lines of non-muscle origin (6 Ewing's sarcomas and 12 osteosarcomas) revealed that the expression of *ET-3* gene was restricted to rhabdomyosarcoma (Fig. 1).

Real-time PCR analysis confirmed the high expression of ET-3 by rhabdomyosarcoma cell lines (Fig. 2B): the expression of ET-3 in rhabdomyosarcoma cell lines was up to 900-fold higher than in normal muscle cells. On the contrary ET-1 expression was poor or absent in rhabdomyosarcoma cells (Fig. 2A), in agreement with the results of conventional RT-PCR (data not shown).

The production of ET-1 and ET-3 at the protein level was analysed by ELISA. All the six human rhabdomyosarcoma cell lines did not show significant level of ET-1 (data not shown) while produced soluble ET-3 ranging from 100 to 400 pg/10^6 cells/ 48 h (Fig. 3A). Moreover, we examined by ELISA the presence of ET-3 in relation to the time course of differentiation of RD/18 and RD/12: this test showed that ET-3 production was independent from the degree of myogenic differentiation reached by the two cell lines (data not shown).

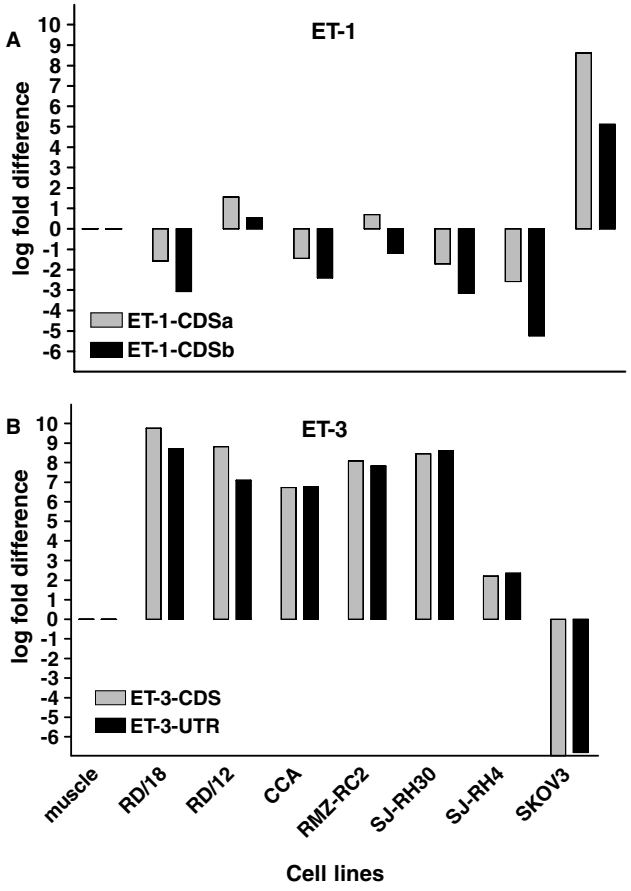

Fig. 2 – Endothelin relative expression (log-fold difference) in rhabdomyosarcoma cell lines evaluated by real-time PCR. The level of ET expression was normalized over that of normal skeletal muscle. SKOV3 is a positive control for ET-1 and negative for ET-3. (A) ET-1 mRNA level was evaluated by two primer pairs: ET-1-CDSa and ET-1-CDSb. (Muscle Ct was 33.5 for ET-1-CDSa and 30.6 for ET-1- CDSb). (B) ET-3 mRNA level was evaluated by two primer pairs: ET-3-CDS and ET-3-UTR. (Muscle Ct was 33.6 for both ET-3-CDS and ET-3-UTR).

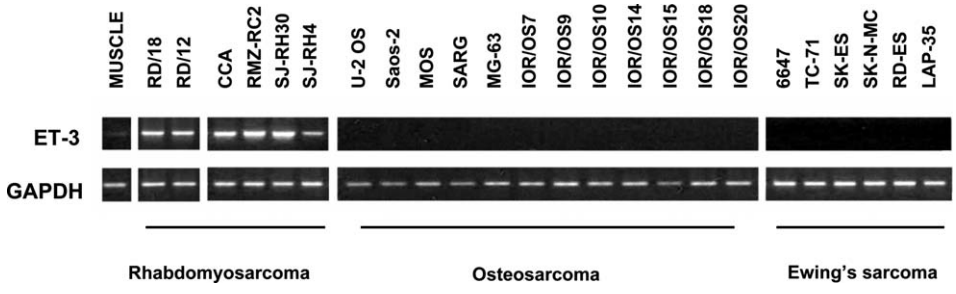

Fig. 1 – *ET-3* gene expression in normal skeletal muscle and human sarcoma cell lines (6 rhabdomyosarcomas, 12 osteosarcomas and 6 Ewing's sarcomas) evaluated by RT-PCR (30 cycles). *GAPDH* (20 cycles) is shown to compare cDNA amounts.

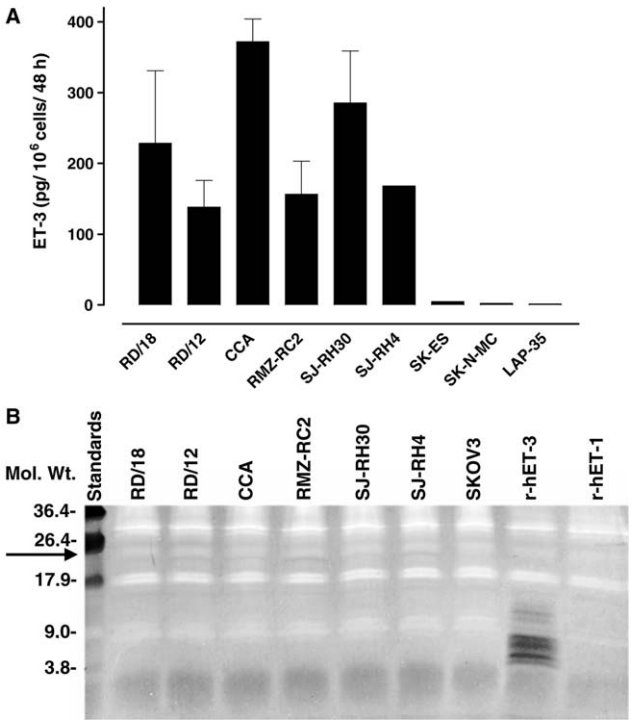

Fig. 3 – ET-3 production analysed in rhabdomyosarcoma cell lines supernatants. (A) ELISA assay. Ewing's sarcoma cell lines were studied as negative controls. Each bar represents the mean ± SE of 2–4 determinations. (B) Western blot analysis. r-hET-3 and r-hET-1 (200 ng each lane) were included to verify the anti-hET-3 antibody specificity. SKOV3 cells were inserted as negative control. Kaleidoscope Polypeptide Standards (Bio-Rad) were used to monitor protein separation during electrophoresis and to assess transfer efficiency on membranes. The arrow indicates the differentially detected protein band (putative ET-3 precursor, 25.454 kDa). Mol. Wt. (molecular weight, Mr in thousands).

The production of ET-3 was further verified by Western blot analysis. Rhabdomyosarcoma cell supernatants showed a protein band that could correspond to the ET-3 precursor (Fig. 3B). In our experimental conditions, we did not detect the ET-3 processed peptide (2.643 kDa) neither in cell conditioned media nor in cell lysates. ET-3 concentration in conditioned media is below the sensitivity of the colorimetric method of detection (reported sensitivity of the Bio-Rad Opti-4CN detection kit: 100 pg).

In order to study the possible existence of an ET-3-based autocrine circuit we investigated the expression of genes coding for the two endothelin receptors by RT-PCR in the rhabdomyosarcoma cell lines and in skeletal muscle. Gene coding for ET_AR was expressed in all cell lines with the exception of SJ-RH4. Amplified product for ET_BR was found only in the alveolar rhabdomyosarcoma cell lines RMZ-RC2 and SJ-RH30 after more than 35 amplification cycles, corresponding to a very low expression (Fig. 4). As most rhabdomyosarcoma cell lines did not express ET_BR and since ET-3 has a low affinity for ET_AR, a significant autocrine role of ET-3 in rhabdomyosarcoma is unlikely.

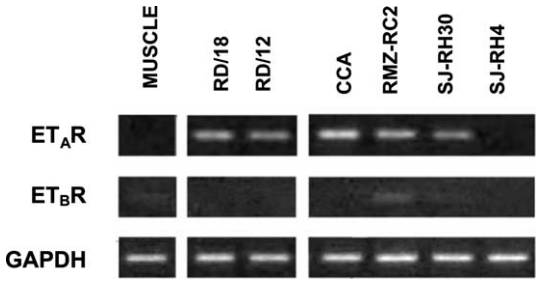

Fig. 4 – Expression of endothelin receptors in normal skeletal muscle and in human rhabdomyosarcoma cell lines by RT-PCR (ET_AR gene, 30 cycles; ET_BR, 35 cycles). The glioblastoma cell line U87MG was used as positive control for both genes (not shown). *GAPDH* (20 cycles) is shown to compare cDNA amounts.

The functional role of ET_AR, present in rhabdomyosarcoma cell lines, was then investigated studying the effect of increasing concentrations of r-hET1 on the growth of rhabdomyosarcoma cells. ET-1 induced a small but dose-related increase in RMZ-RC2 cell growth and produced a small decrease in the other cell lines tested (Fig. 5A). Pretreatment of RMZ-RC2 cells with the ET_AR antagonist BQ123 significantly blocked the r-hET1-induced growth (Fig. 5B). In RMZ-RC2 rhabdomyosarcoma cell line, that weakly expressed ET_BR, the pretreatment with the ET_BR antagonist BQ788 inhibited r-hET-3-induced cell growth increase.

To study the role of possible paracrine circuits of ET-3, we studied whether ET-3 containing supernatants could affect endothelial cell motility. Human umbilical vein endothelial cells (HUVEC), with or without ET_BR antagonist BQ788, were incubated in chemotaxis chambers with RD/18 conditioned medium, for 5 h. We used the selective antagonist in three different concentrations: 1, 10, 100 μM. HUVEC migration toward RD/18 conditioned medium was inhibited in a dose-dependent manner by the ET_BR antagonist (Fig. 6). Migration toward RD/18 conditioned medium was not higher than toward RPMI + 1% FBS control medium; but only the migration induced by RD/18 conditioned medium was specifically inhibited by the ET_BR antagonist BQ788. ET-3, produced by rhabdomyosarcoma cells can play a paracrine role inducing, in vitro, the specific migration of human endothelial cells.

To investigate if ET-3 was expressed also in vivo in human rhabdomyosarcoma tumour samples, we analysed the expression of ET-3 gene on 10 biopsies of human rhabdomyosarcoma (5 embryonal and 5 alveolar) by RT-PCR (Fig. 7). The level of expression of endothelin-3 in all samples indicated that the gene is transcribed also in human tumour specimens in vivo.

Rhabdomyosarcoma expression of ET-3 in vitro and in vivo, and the lack of expression in other musculo-skeletal tumour cell lines suggest that it could be further evaluated as a new molecular marker of rhabdomyosarcoma. Therefore, we studied ET-3 presence in the plasma of mice bearing human rhabdomyosarcoma xenografts. ET-3 was clearly detectable in samples from mice bearing RD/18 tumours, whereas it was absent in samples from non-tumour-bearing nude mice

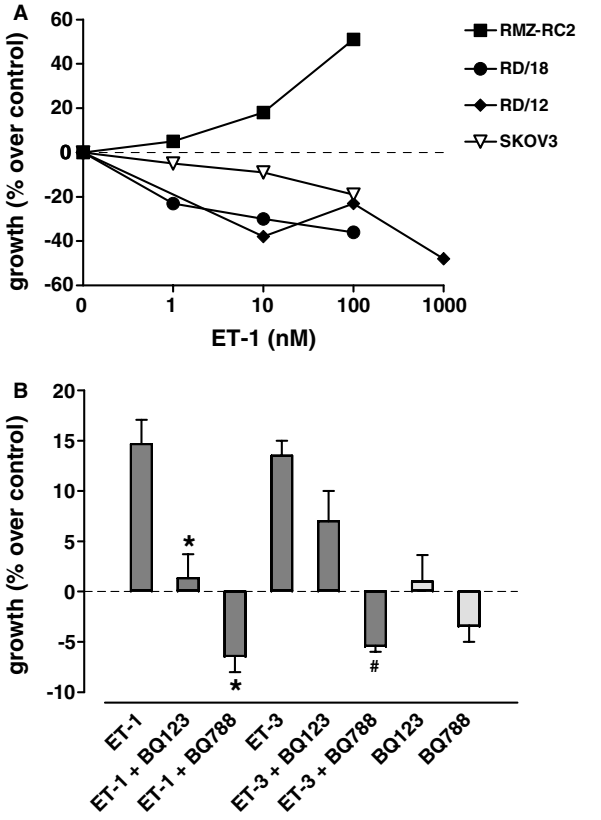

Fig. 5 – Effect of recombinant exogenous endothelins and receptor antagonists on rhabdomyosarcoma cells. (A) r-hET-1 dose response curve in three rhabdomyosarcoma cell lines (RMZ-RC2, RD/18 and RD/12) and in the ovarian carcinoma cell line (SKOV3). Percent of growth over untreated control is shown for each cell line. (B) Effect of pretreatment with ET receptor antagonists BQ123 or BQ788 on the r-hET-1 or r-hET-3-induced growth of RMZ-RC2 cells. Percent of growth over untreated control is shown. Each bar represents the mean ± SE of 2–3 determinations. The results were compared with Student's *t* test ($^{*}P < 0.05$ vs. ET-1 alone, $^{\#}P < 0.05$ vs. ET-3 alone).

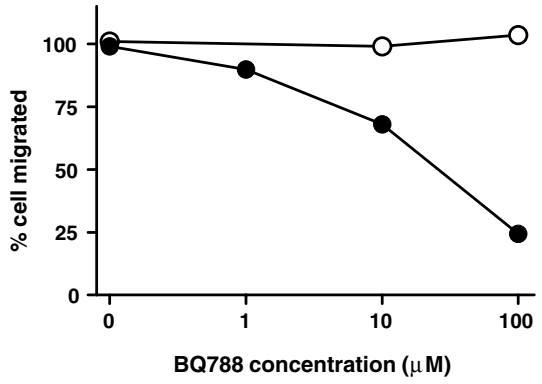

Fig. 6 – Specific inhibition of HUVEC toward RD/18 conditioned medium by the ET_BR antagonist BQ788. Before migration assay HUVEC cells were pretreated with increasing concentrations of BQ788. Open symbols show migration toward control medium RPMI + 1% FBS, closed symbols show migration induced by RD/18 conditioned medium.

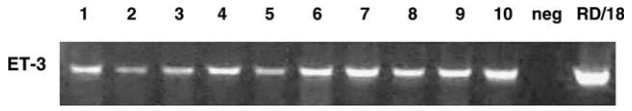

Fig. 7 – ET-3 gene expression by RT-PCR (40 cycles) in biopsies of human rhabdomyosarcoma (1–10). RD/18 cell line was used as positive control.

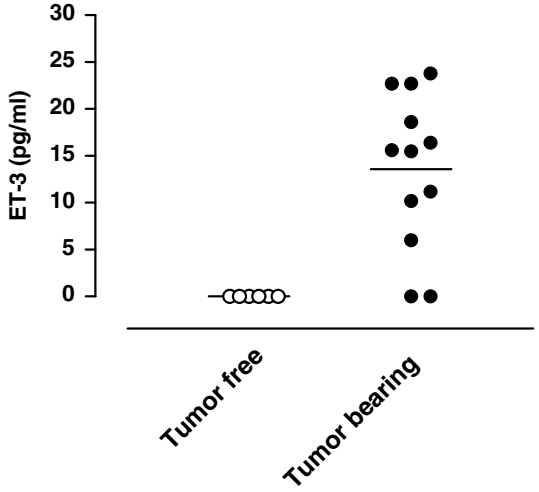

Fig. 8 – ET-3 plasma levels in mice bearing human rhabdomyosarcoma. ELISA assay was performed on plasma from individual nude mice with tumours originated from subcutaneous RD/18 cell injection (closed symbol). Plasma levels in control nude mice are reported for comparison (open symbol).

(Fig. 8). This suggest that plasmatic ET-3 could be investigated as a new marker of rhabdomyosarcoma.

4. Discussion

We demonstrated that the small vasoactive peptide ET-3 is expressed by different human rhabdomyosarcoma cell lines and tumours, whereas it is absent in other sarcomas like osteosarcoma and Ewing's sarcoma.

ET-3 is part of the endothelin system that also includes ET-1 and ET-2, and their receptors ET_AR and ET_BR. Recent work in carcinomas and other tumour types by Bagnato and Natali[12] highlighted the role of ET-1 and ET-2 and the $ET_{A/B}R$ axis. We found that rhabdomyosarcomas do not express or poorly express either ET-1 or ET_BR, thus ruling out the possibility of a significant autocrine endothelin loop in this type of tumour. In RMZ-RC2 cells, a very weak mitogenic effect by endogenous or exogenous ET-3 is suggested by the effect of ET_BR antagonist BQ788 pretreatment. Furthermore RMZ-RC2 cells showed a different growth trend in response to ET-1 in comparison to the other cell lines. A role for ET_BR in ET-1 cell growth increase in RMZ-RC2 cells could also be suggested by data obtained with ET_BR antagonist BQ788.

Expression of ET-3 in rhabdomyosarcoma suggests that ET-3 might be involved in neoplastic growth of muscle cells. Indeed we also found that clinical samples shared with rhabdomyosarcoma cell lines the expression of ET-3. A possible role of ET-3 in the biology of rhabdomyosarcoma is the interaction with endothelial cells for the angiogenic switch required for tumour growth. We found that ET-3 released by rhabdomyosarcoma cells induced HUVEC motility.

Other studies demonstrated that ET-1 and ET-3, acting through the ET_BR, have dose-dependent stimulatory, proliferative and migratory effects on HUVEC.[11,26–28] The results of migration assay induced by rhabdomyosarcoma conditioned medium and the specific inhibition obtained with ET_BR antagonist BQ788 are in agreement with the role played by ET_BR on HUVEC motility.

The interaction between ET-1 and ET_BR has been reported to play a role in tumour neovascularization.[29–31] So it is possible that, in rhabdomyosarcoma, ET-3 substitutes ET-1 in its angiogenic function. The pro-angiogenic effect could be a direct consequence of binding between ET-3 and its receptor; otherwise ET-3 could enhance the production of vascular endothelial growth factor that induce a higher expression and secretion of ET-1.[14] Therefore we can conclude that ET-3 produced by human rhabdomyosarcoma is a peptide with a paracrine action on endothelial cells and could be investigated as a target for anti-angiogenic therapies against this type of tumour. Rhabdomyosarcoma produces other factors with paracrine action that might promote angiogenesis in collaboration with ET-3. Stem Cell Factor is a growth factor with multiple functional roles in development and it is expressed by human rhabdomyosarcoma cell lines while its receptor c-kit is absent.[8] Stem Cell Factor has probably a paracrine action on tumour neighbouring tissue and could be involved in tumour vascularization: this cytokine, expressed by mammary tumour cells, has profound effects on tumour angiogenesis via the modulation of mast cell activities.[32]

Our results indicate that ET-3 could be investigated for clinical uses in rhabdomyosarcoma. We found that nude mice bearing human rhabdomyosarcoma xenografts had readily detectable ET-3 plasma levels, therefore ET-3 might be further investigated as a possible serological marker for diagnostic purposes and for patient follow-up. An even more interesting development of ET-3 would be as a therapeutic target for the inhibition of neoangiogenesis.[12]

Conflict of interest statement

None declared.

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