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

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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

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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]