Matrix-mediated canal formation in primmorphs from the sponge *Suberites domuncula* involves the expression of a CD36 receptor-ligand system

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Summary

Sponges (Porifera), represent the phylogenetically oldest metazoan phylum still extant today. Recently, molecular biological studies provided compelling evidence that these animals share basic receptor/ligand systems, especially those involved in bodyplan formation and in immune recognition, with the higher metazoan phyla. An in vitro cell/organ-like culture system, the primmorphs, has been established that consists of proliferating and differentiating cells, but no canals of the aquiferous system. We show that after the transfer of primmorphs from the demosponge *Suberites domuncula* to a homologous matrix (galectin), canal-like structures are formed in these 3D-cell aggregates. In parallel with the formation of these structures a gene is expressed whose deduced protein falls into the CD36/LIMPII receptor family. The receptor was cloned and found to be strongly expressed after adhesion to the galectin matrix. This process was suppressed if primmorphs were co-incubated with a homologous polypeptide containing the CSVTCG domain, as found in thrombospondin-1 (and related) molecules of vertebrates.

Key words: Sponges, Porifera, *Suberites domuncula*, CD36, Lysosomal integral membrane protein II, Thrombospondin, 2-methylthio-1,4-naphthoquinone, Canal formation, Angiogenesis

Introduction

Sponges (Porifera), as the phylogenetically oldest metazoan phylum still extant today, are the closest relatives of the hypothetical common metazoan ancestor, the Urmetazoa (reviewed by Müller, 2001). Compelling evidence has accumulated during the last few years, especially through cloning of genes and subsequent analysis of their functional roles, that sponges possess the basic structural and functional elements that allow a tuned interaction of their differentiated cells (see Müller, 1997). In particular, the immune molecules and the recently identified apoptotic enzymes and receptors enabled sponges to pass the transition from the colonial stage of organization to an integrated stage (Müller, 2003). Intracellular transcription factors, e.g. the homeodomain molecules such as the LIM homeodomain factors (Wiens et al., 2003) and extracellular matrix molecules with their interacting surface-associated receptors, e.g. integrin and their ligands (Wimmer et al., 1999; Schütze et al., 2001), provided the molecular platform for the development of a bodyplan (Wiens et al., 2003). The tuned interactions of the differentiated cells, controlled by diverse regulatory molecules, allowed a pattern formation.

Even though nerve cell-like receptors have been identified in sponges (Perović et al., 1999), no nervous system can be detected in Porifera; likewise sponges do not possess a blood circulating system. Nevertheless sponges have a complex water circulating system connecting inhalant and exhalant canals (Möhn, 1984). Sponges require this aquiferous system for their supply of nutrients (Simpson, 1984) and of oxygen (Gatti et al., 2002). Recent studies demonstrated that the speed of the water current surrounding the animals induces morphogenetic processes. These studies were performed with primmorphs from the demosponge *Suberites domuncula* (Custodio et al., 1998; Müller et al., 1999). Primmorphs are a
expression of a homeobox gene, Iroquois (Perovic et al., 2003).

Primmorphs can be used as biofermenters for the production of bioactive secondary metabolites (Müller et al., 2000). So far their size is limited to approximately 20-40 mm, however, one possibility for increasing their size is to induce canal formation by culturing them on a homologous matrix. We demonstrated that primmorphs form canal-like structures with the S. domuncula galectin as a matrix (Wiens et al., 2003). Also it is known that growth of mammalian tumors depends on the formation of new capillary blood vessels, as first formulated in 1971 by Folkman (Folkman, 1971). One powerful technique to study angiogenesis in vitro is the use of the 3D-cell cultures, the spheroids (reviewed by Müller-Klieser, 1997). With those spheroids it could be shown that angiogenesis depends on soluble factors, e.g. vascular endothelial growth factors (VEGF) or cytokines, which interact with the corresponding receptors localized on the endothelial cells, e.g. the VEGF receptor (see Brower, 1999).

In an attempt to understand, in molecular terms, the transition of the two growth forms of the sponge primmorphs/aggregates (from spheroid to flat; ball-like to canal-like forms) we investigated mechanisms in sponges that have been previously described in vertebrates. All known receptors and their ligands involved in the genesis of new blood vessels in vertebrate systems are restricted to Metazoa. In the present study we examined whether the CD36-thrombospondin (TSP) system, which is assumed to be phylogenetically old (Calvo et al., 1995) is also involved in canal formation of primmorphs.

The glycoprotein CD36, initially known as glycoprotein IV was first identified biochemically on the platelet membranes (Okumura and Jamieson, 1976) and subsequently was studied at the gene level (Wyler et al., 1991). Together with the membrane glycoprotein CD36/LIMPII analog-1 (CLA-1) (Calvo and Vega, 1993) and the lysosomal integral membrane protein II (LIMPII) (Vega et al., 1991), these molecules form the CD36 family (Calvo et al., 1995). Based on a calibrated molecular clock it was estimated that the ancestor of these molecules emerged around 850 to 650 million years ago (Calvo et al., 1995). The CD36 molecules, as well as the LIMPII transmembrane proteins, function as receptors for the extracellular protein thrombospondin (TSP) (Crombie and Silverstein, 1998). TSP-1 is a 450 kDa trimeric glycoprotein that is a ligand for the CD36 receptor (Asch et al., 1987) and also binds to LIMPII (Crombie and Silverstein, 1998). While it is well established that CD36 is a cell surface receptor (Greenwalt et al., 1992) known to bind to collagen (see Enenstien et al., 1998), LIMPII is integrated into the lysosome membrane (Vega et al., 1991). Since a series of lysosomal receptors, e.g. LAMP-1 or LAMP-2, can be found on activated platelets (Silverstein and Febbraio, 1992) or tumor cells and leukocytes (Fukuda, 1985), it was not surprising that LIMPII also binds to TSP-1 (Crombie and Silverstein, 1998) with the CSVTCG peptide segment that is located within the type 1 repeats of TSP (Li et al., 1993).

The interaction of CD36 members with TSP-1 results in morphogenetic processes and at the same time modulates/inhibits proliferation and differentiation as well as angiogenesis and cell migration on endothelial cells (Dawson et al., 1997). Prevention of the interaction of CD36 with TSP-1 is one target for an anti-angiogenic tumor therapy (Brower, 1999). Besides natural endogenous inhibitors and the recently discovered metallopondins or synthetic inhibitors, thrombospondin is also considered as a good drug candidate for the ‘starvation’ of a tumor (Brower, 1999). Recently, also secondary metabolites from sponges, which are characterized by highly diverse chemical structures (reviewed by Sarna et al., 1993) causing powerful and specific bioactivities (Faulkner, 2000), have been found to display anti-angiogenic activity. Among them is aeropylin-1, an inhibitor isolated from the sponge Verongia aerophoba, which inhibits the epidermal growth factor receptor tyrosine kinase (Kreuter et al., 1990) and also displays anti-angiogenic activity at micromolar concentration (Rodrigues-Nieto et al., 2002).

In the present study we describe how during transformation of round sponge primmorphs to their flat attached growth form with canal-like structures, the CD36/LIMPII receptor is increasingly expressed. This process could be prevented by addition of a polypeptide (TSP-1) consisting of the binding domain to CD36/LIMPII. CD36/LIMPII was cloned and expressed from the S. domuncula cDNA. Furthermore, we describe a compound that was isolated from a sponge-associated bacterial strain within our screening program aimed at the identification of novel bioactive natural products in marine organisms by hyphenated HPLC techniques (Bringmann and Lang, 2003). This compound, 2-methylhexio-1,4-naphthoquinone (MTN), inhibits canal formation in primmorphs. MTN is a naphthoquinone derivative hitherto known only as a synthetic product (Fieser and Brown, 1949). Sulfur-containing naphthoquinones, but equipped with additional prenyl residues in the 3-position, have been isolated from a thermophilic hydrogen-oxidizing bacterium (Ishii et al., 1987). MTN has been found to exhibit antifungal (Gershon and Shanks, 1975) and antitumor activity (Takano et al., 1960).

Materials and Methods
Chemicals and enzymes
The sources of chemicals and enzymes have been described earlier (Kruse et al., 1997; Wimmer et al., 1999; Krasko et al., 2000).

Sponges
Live specimens of S. domuncula (Porifera, Demospongeia, Hadromerida) were collected near Rovinj (Croatia) and subsequently were kept in aquaria in Mainz for more than 4 months prior to use (Le Pennec et al., 2003).

Dissociation of cells and formation of primmorphs
The procedure described for the formation of primmorphs from single cells was applied (Custudio et al., 1998; Müller et al., 1999). Starting from single cells, primmorphs of 3-7 mm are formed after 5 days. For the experiments described here 6-day-old primmorphs were used. They were cultured in natural seawater supplemented with 0.2% of RPMI1640 medium and with the optimal concentration of silicate (60 μM) and Fe3+ (30 μM, added as ferric citrate) (Krasko et al., 2002).

After primmorph formation, these three-dimensional aggregates...
were transferred to 12-well tissue culture test plates (Nunclon-
Surface; Nunc, Wiesbaden; Germany; TPP) either uncoated or coated
with recombinant homologous galectin. For coating, 500 µl of a
recombinant galectin solution (15 µg/ml) were added per well. After
standing for 12 hours at 4°C the plates were washed with seawater
and used for the experiments. Subsequently incubation was prolonged
for an additional 6 days. During this period the recombinant TSP
peptide or MTN was added, as indicated.

In one series of experiments the recombinant TSP peptide was pre-
incubated with the recombinant 15 kDa CD36/LIMPII receptor
polyepitope for 3 hours (21°C) prior to the addition to the primmorphs
culture.

S. domuncula galectin cDNA and the recombinant protein
The complete cDNA encoding galectin was isolated from S.
domuncula applying the polymerase chain reaction (PCR) technique
(Wiens et al., 2003). The cDNA had a size of 955 nt (excluding the
poly(A) tail). The clone was termed SDGALEC1 (accession number
AJ493055). For the synthesis of recombinant galectin the SDGALEC1
cDNA was cloned into the pGEX2T plasmid containing the
Schistosoma japonicum glutathione S-transferase (GST) gene.
Expression was induced with isopropyl β-D-thiogalactopyranoside
(IPTG) for 24 hours. The GST fusion protein, termed
goLIMPII_SUBDO, was purified by affinity chromatography on
glutathione Sepharose 4B (Coligan et al., 2000). The fusion protein
was cleaved with thrombin (10 units/mg) to separate glutathione S-
transferase from the recombinant sponge galectin; the size of the
purified galectin has a molecular mass of 22 kDa. This material was
used for coating of the cultivation plates.

Isolation of the cDNA encoding the S. domuncula CD36/LIMPII
receptor
The complete cDNA encoding the CD36/LIMPII receptor, SSDCD36/LIMPII, was isolated from the cDNA library from S.
domuncula (Kruse et al., 1997) by PCR technique. The primer was
designed against the conserved amino acid residues, found in the
‘block A’ segment of the lysosomal integral membrane protein II
(LIMPII) (Crombie and Silverstein, 1998). The degenerate forward
primer against aa92 to aa101 (human LIMPII; accession number
NP_005497.1 [Calvo et al., 1995]), 5’-GAA/G GAA/G GTA/T/G/C
GGG/T/G/C CCA/G/T/C TAC/T-3’, was used in the PCR reaction
together with the vector primer. The PCR conditions were as follows.
Initial denaturation at 95°C for 3 minutes, followed by 30
amplification cycles at 95°C, for 30 seconds, 56°C for 45 seconds,
74°C for 1.5 minutes, and a final extension step at 60°C for 10
minutes. The reaction mixture was as described earlier (Wiens et al.,
1998). One fragment of ~1200 bp was isolated and used to complete the
cDNA sequence by screening the library (Ausubel et al., 1995).
The clone was termed SCD36/LIMPII.

Recombinant S. domuncula CD36/LIMPII receptor
To prepare a recombinant part of the CD36/LIMPII receptor, the
sp cons cDNA (SDCD36/LIMPII) corresponding to the first 131 aa,
including the characteristic domain ‘block A’ of the predicted protein
(open reading frame) was cloned into the pQE vector (Quiagen,
Hilden, Germany) and used to transform BL21 strain bacteria, as
successfully used previously for the preparation of recombinant
sp sponge proteins (Adell et al., 2003). One positive colony was selected
and induced with IPTG for 4 hours at 37°C; cells were collected and the
expressed r-CD36/LIMPII was purified on a Ni-NTA agarose
column (Quiagen), according the manufacturer’s instructions. The
eluted fraction containing the r-CD36/LIMPII protein was dialyzed
against seawater and used for the incubation/competition studies. The
size of the polypeptide, 15 kDa, was verified by gel electrophoresis
in the presence of 12% polyacrylamide containing 0.1% NaDodSO4
(data not shown).

Northern blotting
RNA was extracted from liquid-nitrogen pulverized sponge tissue
with TRIzol Reagent (Gibco-BRL, Grand Island, NY). Then 5 µg
total RNA was electrophoresed through a 1% formaldehyde/agarose
gel and blotted onto Hybond-N+ nylon membrane following the
manufacturer’s instructions (Amersham, Little Chalfont,
Buckinghamshire, UK) (Wiens et al., 1998). Hybridization was performed
with 0.5 kb segments of the SDCS6/LIMPII cDNA, the
galectin (SDGALEC1) cDNA or the SDADAMTS cDNA. The probes
were labeled with the PCR-DIG-Probe Synthesis Kit according to the
‘Instruction Manual’ (Roche). After washing, digoxigenin (DIG)-
labeled nucleic acid was detected with anti-DIG Fab fragments
(conjugated to alkaline phosphatase; dilution of 1:10,000) and
visualized by chemiluminescence technique using CDP, the
di-chloromercuribenzenesulfonate substrate alkaline phosphatase, according to the
instructions of the manufacturer (Roche). The signals of the blots were
quantified by applying the chemiluminescence procedure of Stanley
and Kricka (Stanley and Kricka, 1990); CDP-Star was used as the
substrate. The screen was scanned and quantified with the GS-525
Molecular Imager (Bio-Rad, Hercules, CA, USA).

In situ localization of CD36/LIMPII receptor
The method of in situ localization applied was based on the procedure
described by Polak and McGee (Polak and McGee, 1998) with
modifications (Le Pennec et al., 2003; Perovic et al., 2003). In brief,
siliceous spicules were removed from tissue samples with HF/NH4F.
After washing with Ca2+- and Mg2+-free artificial seawater (CMFSW)
(Rottmann et al., 1987) the sponge pieces were embedded in Tissue-
Tek. In contrast to the tissue materials, the primmorphs were not
reated with HF/NH4F but were directly embedded into Tissue-Tek.
8-μm sections through frozen tissue and primmorphs were cut.
Cyrosections were fixed with paraformaldehyde (4% in CMFSW) for
30 minutes, and then washed with phosphate-buffered saline (PBS) at
room temperature. Hybridization was performed in 2× SSC (sodium
carbonate/sodium citrate), supplemented with 50% formamide.
The activation of the DIG-labeled probe (10 pmol/ml) was performed for
1 minute at 95°C in 4× SSC buffer. To each cryosection, 65-70 µl of
hybridization buffer, including the probe, was added. Hybridization
proceeded overnight in a glass chamber at 45°C. Subsequently the
sections were washed at 55°C, 1 minute in 2× SSC and 5× 20
minutes in 0.2× SSC. Final washes (2× 5 minutes) were with
PBS containing 0.1% (v/v) Triton X-100 and 2 mg/ml of BSA at
room temperature. After blocking [1% blocking reagent for nucleic
acids (Roche) in 1× PBS containing 0.1% (v/v) Tween 20] for 15
minutes at room temperature, the slices were reacted with anti-DIG-
Fab fragments conjugated to alkaline phosphatase (dilution 1:100) for
1 hour at 37°C in a humid chamber. After two washes (5 minutes each)
with PBT at room temperature and one wash (5 minutes) with the Tris
buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl and 50 mM MgCl2),
the sections were incubated with Tris buffer, supplemented with the
dye reagents NBT (4-nitro blue terazolium chloride) and X-phosphate
(Roche) for 45 minutes at 37°C in the dark. After washing the sections
for 5 minutes in 1× PBS, they were covered in Glycergel (DAKO,
Hamburg, Germany) and analyzed under the microscope.

The DNA probe had a length of 450 bp (nt1150 to nt1630 within the
cDNA) and comprised the 3'-terminal region of the ORF of the
SDCD36/LIMPII cDNA. PCR was carried out with an initial
denaturation at 95°C for 3 minutes, then 35 amplification cycles each
at 95°C for 30 seconds, 58°C for 30 seconds, 74°C for 4 minutes, and
a final extension step at 72°C for 20 minutes. Labeling was performed
by using the DIG Oligonucleotide Labeling Kit (Roche). The
antisense-SDCD36 probe was found to react with cells in the section,
while the sense-SDCD36 probe, synthesized complementary to the direction of the anti-sense probe, showed no binding. The sections were inspected with a Olympus VANOX AHB T3 microscope. The images were recorded with a ColorView 12 camera, applying the Soft Image System analySIS 3.0 (Soft Image System GmbH, Münster, Germany).

Cloning and expression of the CSVTCG cell adhesive motif

Searching the expressed sequence tag (EST) cDNA database of *S. domuncula*, which had been established by our group, several ESTs were detected that had high sequence similarity to the ADAMTS protein, also termed disintegrin/metalloproteinase with thrombospondin motifs. One sequence of 541 nucleotides was extracted and termed *SDADAMTS*; it contained no stop codon. A 348 nt fragment from base pairs 2-349 (see below) was subcloned into the bacterial glutathione S-transferase/oligohistidine/S expression vector pET41a (Novagen, Madison WI, USA) via the Xho I (3’ end) and the Eco RV (5’ end) restriction sites. E. coli, strain BL21, was transformed with this plasmid and expression of fusion protein was induced for 6 hours at 37°C with 1 mM IPTG (Ausubel et al., 1995).

Bacteria from 500 ml cultures were obtained by centrifugation; the induced for 6 hours at 37°C with 1 mM IPTG (Ausubel et al., 1995). The rDNA analysis revealed that D1 shares 99% identity to the alpha-rDNA of *Proteus vulgaris* (accession number AF218241). The bacterial strain D1 was cultured and extracted with n-butanol and the *SDCD36*¢ end) restriction sites.

**Sequence analysis**

The sequence was analyzed using the BLAST (2003) and FASTA (2003) computer programs. Multiple alignments were performed with CLUSTAL W Ver. 1.6 (Thompson et al., 1994). Phylogenetic trees were constructed on the basis of amino acid sequence alignments by neighbor-joining, as implemented in the Neighbor program from the PHYLP package (Felsenstein, 1993). The distance matrices were calculated using the Dayhoff PAM matrix model as described previously (Dayhoff et al., 1978). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein, 1993). The graphic presentations were prepared with GeneDoc (Nicholas and Nicholas, 2001).

**Isolation and characterization of 2-methylthio-1,4-naphthoquinone (MTN)**

A tissue sample from the central core of the sponge *Dysidea avara* was stanced out under sterile conditions and rinsed 3-4 times with sterile seawater. This tissue was squeezed between two sterile glass slides. The resulting extract was further diluted (10-1 to 10-5) and a sample of each dilution was plated on agar medium (0.25% peptone, 0.15% yeast extract, 0.15% glycerol, 1.6% agar, 100% seawater). The plates were incubated at 30°C for 24-72 hours. Bacteria were further purified and cultured on agar medium. Eight bacterial strains (D1-D8) were obtained from this sponge specimen.

The bacterial strain D1 was cultured and extracted with n-butanol following the method of Elyakov et al. (Elyakov et al., 1996). 16S rDNA analysis revealed that D1 shares 99% identity to the alpha-proteobacteria MBIC3368 (accession number AF218241). The bacterial isolate was inoculated into conical flasks (1 liter) with 500 ml cultural broth. The culture broth contained peptone (0.25%), yeast extract (0.15%) and glycerol (0.15%) in seawater. The pH was adjusted to 7.2-7.5. The flasks were incubated at 30°C for 3 days with shaking (100 rpm). After incubation, bacterial cultures [500 ml each (total 2 l)] were mixed with 150 ml of n-butanol. The mixture was kept at 40°C for 24 hours, stirred for 20 minutes, centrifuged and the butanol layer was separated and then evaporated using a rotary evaporator. Dry residue (100-150 mg) was stored below 5°C until further use.

The extract was subjected to gel filtration on Sephadex LH-20 (23x3 cm; eluent methanol; fraction size, 18 ml). Fractions 14 and 15 were evaporated to dryness and further purified using preparative high-performance liquid chromatography (column: Waters Xterra 19×300 mm; solvents: water and acetonitrile, both with 0.05% TFA; gradient: 0 minutes – 10% acetonitrile, 30 minutes – 100% acetonitrile; flow rate: 12 ml/minute). The compound that was eluted after 23 minutes yielded, after drying, 0.5 mg of pale yellowish needles. The substance was identified as 2-methylthio-1,4-naphthoquinone (MTN) by evaluation of NMR and MS spectra: the data show good agreement with those published for MTN (Kametani et al., 1977; Coll et al., 1988). The melting point (mp) was determined to be 176-178°C (Kametani et al.: mp 165-166°C; Coll et al.: mp 185-186°C).

**Cytotoxic activity**

Four permanent mammalian tumor cell lines were applied to determine the cytotoxic effect caused by MTN: murine leukemic lymphoblasts L5178y (ATCC CRL 1722), rat adrenal pheochromocytoma cells PC-12 (ATCC CRL 1721), human cervix HeLa S3 cells (ATCC CCL 2.2) and the rat embryonal fibroblast cells Rat1 (Klock et al., 1998). The tumor cells were grown as described previously (Müller et al., 1977). The cell viability assay was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) method as described previously (Taglialetela et al., 1977). The cells were seeded in developing embryo and a window of 1 cm² was marked. The marked eggs were ‘candled’ so as to locate the position of developing embryo and a window of 1 cm² was marked. The marked window was cut in order to see the embryo and the surrounding blood vessels on CAM. An agar disc impregnated with a known concentration of the compound was placed in the outer third portion of the CAM, in the region of the proliferative capillaries-dendrites. After placing the disc on the CAM, the window was resealed with Parafilm and the eggs were incubated at 38°C for 48 hours. After incubation, eggs were opened and the anti-angiogenic response was assessed by measuring the avascular zone of the CAM beneath the disc. A positive control of a mixture of hydrocortisone (60 µg) and heparin (50 µg) was applied, which showed 100% anti-angiogenic activity. An agar discs with saline was used as a negative control, which did not show activity. At least 20 eggs were used for each dose and the experiments were performed in triplicate to ensure reproducibility.
in 96-well plates at a density of 10×10⁴ cells per well with or without the compound. After 72 hours, plates were read on the microplate reader (model 450; Bio-Rad) at a test wavelength of 595 nm. Ten parallel assays were performed for every concentration of MTN. The ED₅₀ was determined by logit regression (Sachs, 1974).

Results

Canal formation in primmorphs

As outlined previously (Wiens et al., 2003), canal formation was induced in primmorphs from *S. domuncula* by cultivating them on a galectin-coated matrix. The primmorphs were obtained from dissociated single cells (Fig. 1A) in seawater, supplemented with RPMI, silicate and Fe³⁺, as described in Materials and Methods. After 3 days aggregates formed that still had a rough surface, but are not composed of the characteristic pinacocyte layer (Fig. 1B) (Le Pennec et al., 2003). However, after 5 days a smooth surface was formed around the non-attached primmorphs, which is composed of pinacocytes (Fig. 1C). After 6 days, the primmorphs remained either in the uncoated plates for an additional 6 days (Fig. 1F), in which case they did not change their spherical appearance and remained non-attached, or, they were cultured on galectin-coated plates for a subsequent 6 days, when the primmorphs attached to the surface, flattened and formed canal-like structures (Fig. 1D,E).

*S. domuncula* CD36/LIMPII receptor

To determine if an expression of the CD36/LIMPII receptor occurs during the canal formation the respective cDNA had to be cloned by PCR. As described in Materials and Methods one degenerate primer was designed against the conserved domain block A (Crombie and Silverstein, 1998). The fragment of the cDNA was obtained and termed *S*.*CD36/LIMPII*; its deduced amino acid (aa) sequence characterizes it as a member of the CD36 family with the CSVTCG sequence, known to interact with the ADAMTS protein (Calvo et al., 1995) proposed that the CD36 family might have an ancestor that existed prior to the protostomian-deuterostomian split, around 650 to 850 million years ago. Therefore, the sponge CD36/LIMPII receptor was compared with the other known members from human and demonstrated in a phylogenetic, slanted cladogram (Fig. 2C); the identity/similarity scores are approximately 20%/40%. With the sponge CD36/LIMPII receptor as an outgroup the next closest related molecule is LIMPII followed by the other members of the family including glycoprotein CLA-1 and members of the family including glycoprotein CLA-1 and CD36. This branching order supports the prediction (Calvo et al., 1995).

CSVTCG-containing peptide

An EST/cDNA that encodes a putative protein with similarity to the ADAMTS protein (*SDADAMTS*) was selected from the database of *S. domuncula*. This molecule contains in the deduced polypeptide the CSVTCG sequence, known to interact both with the CD36 receptor and the LIMPII receptor (Li et al., 1993; Crombie and Silverstein, 1998). The fragment of the deduced sponge protein has high sequence similarity to the...
Expression of the CD36/LIMPII receptor gene in primmorphs

Northern blotting was applied to semi-quantitatively measure the expression of the gene encoding the CD36/LIMPII receptor. After extraction of RNA and application of the same amount of RNA per sample onto the gel it became obvious that the single cells (Fig. 5A; time 0), as well as the primmorphs that were cultured on uncoated dishes for 6 days, showed only a low expression of the CD36/LIMPII receptor gene; the transcript size was 1.7 kb. However, if the primmorphs were transferred after 6 days into culture dishes that had been coated with galexin, a strong increase of expression was seen. The highest steady-state expression of 5.1-fold increase (compared to the expression measured at day 0) was seen 3 days after the transfer (total incubation period of 9 days); almost the same level of expression (4.5 fold) was seen after an incubation period of 6 days on the galexin matrix (Fig. 5A).

In contrast, if the primmorphs were left on uncoated dishes for an additional 6 days after the initial incubation period, they remained unattached and did not show marked changes in the expression of the respective genes; the expression was estimated to be 1.9-fold higher than the control level. Interestingly the primmorphs that were transferred after 6 days onto galexin-coated dishes and were treated additionally with thrombospondin motifs (ADAMTS). The polypeptide used here was most similar to ADAMTS-9 (Q9P2N4); it shared 20% identical and 30% similar aa with the human ADAMTS-9 precursor (Clark et al., 2000) (Fig. 3). A stretch of 348 nt within SDADAMTS was selected from the cDNA for the preparation of the recombinant polypeptide. The 116 aa long peptide has a calculated size of 12,749 Da (Fig. 4).
respect to the expression level seen in the absence of the primmorphs, incubated with SDADAMTS alone show a low CD36/LIMPII receptor. Subsequently, a northern blot had been pre-incubated with equal amounts of recombinant SDADAMTS only or with the recombinant SDADAMTS that outcome.

In one series of experiments the primmorphs (developed on S. domuncula galectin in primmorphs; after attachment to the substrate the primmorphs formed canal-like structures. Therefore, it was advisable to determine those cells that express high levels of transcripts encoding the CD36/LIMPII receptor. Recently we established the technique of in situ hybridization for sponge tissue/primmorphs (Perović et al., 2003), which we applied for this purpose. By this approach with the antisense SDCD36/LIMPII cDNA as a probe it was found that only cells that surround the aquiferous canals within the sponge tissue could be stained (Fig. 6A,C). In contrast, the sense SDCD36/LIMPII probe did not react with any sponge cells (Fig. 6B,D).

Isolation and characterization of MTN
The extract of a sponge-associated bacterium (an alphaproteobacterium) isolated from the sponge D. avara exhibited considerable anti-angiogenic activity in the CAM assay. Therefore, the active compound was isolated using gel filtration on Sephadex LH-20 and reversed-phase preparative HPLC in a bioactivity-guided manner. The compound thus obtained was readily identified using MS and NMR spectroscopy as 2-methylthio-1,4-naphthoquinone (MTN; Fig. 7).

Anti-angiogenic activity using the CAM assay
To test the compound for anti-angiogenic activity in vivo, the chick chorio-allantoic membrane (CAM) assay was applied (Auerbach et al., 2003).

Our studies reveal that MTN is a potent angiogenesis inhibitor. It showed 100% activity at a concentration 1 ng/disc. This compound causes discontinuous and disrupted blood vessels in a wide area around the disc. The normal embryogenesis pattern of the chick embryo is shown in Fig. 8A. However, if MTN was added at a dose of 0.25 ng (per disc) (Fig. 8B), or of 1 ng (Fig. 8C), a distinct avascular zone at the place of application is seen. A quantitative analysis showed that at a dose of 0.25 ng (per disc) 20% of the embryos showed a significant anti-angiogenic effect (n=20); at the higher doses of 0.50 ng to 1 ng (per disc) of MTN, the percentage of embryos with a significant anti-angiogenic effect reached 100%. The effect of MTN on the viability of L5178y leukemic
L5178y cells were inhibited by MTN with an ED50 value of 0.08±0.02 μg/ml. The effect on the other tumor cells was less strong: the ED50 value was determined to be > 0.3 μg/ml.

Effect of MTN on the expression of the CD36-like receptor in *S. domuncula*

Northern blot analysis was applied to determine if MTN also causes a modulating effect on the expression of the CD36/LIMPII receptor gene in the sponge primmorph system. Primmorphs cultured for 3 days on the galectin matrix in the presence of a low concentration of MTN showed a strong reduction of the steady-state level of this gene (Fig. 5).

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MTN

2-methylthio-1,4-naphthoquinone
Reduction of expression of the CD36-like receptor in primmorphs by ADAMTS and MTN

The expression of the CD36/LIMPII receptor gene was analyzed after treatment with ADAMTS and MTN by in situ hybridization. The expression was monitored in primmorphs grown on the galectin matrix. An intense staining of the cells, especially around the canal-like structures (c) is seen. (D,E) Primmorphs that had been cultured on galectin but in addition either in the presence of 10 μg/ml of the recombinant SDADAMTS (D), or 0.03 μg/ml of MTN (E). After the 3 days incubation the primmorphs were analyzed by in situ hybridization with the antisense probe SDCD36/LIMPII. Magnifications: (A,B) ×5; (C-E) ×15.

Expression of ADAMTS in primmorphs in dependence on MTN

Finally, the expression of SDADAMTS in primmorphs was determined. Northern blot experiments show that the expression of SDADAMTS is strongly downregulated in primmorphs cultured on the galectin matrix. The expression value drops to 0.2 fold compared to the expression measured at day 6 of incubation in uncoated dishes (Fig. 5D). This low level of expression remained unchanged if the primmorphs were co-incubated with 0.01 μg/ml or 0.01 μg/ml of MTN (Fig. 5D).

Discussion

Until recently the factors controlling the induction and formation of the complex aquiferous canal in sponges were unknown. With the elucidation that sponges, such as S. domuncula, can express homeobox genes, e.g. Iroquois, in response to extracellular conditions of the aqueous milieu (Perović et al., 2003), it became clear that oxygen has also a morphogenetic function. Having established that the Iroquois transcription factor is, after its induction through oxygen, crucial for the formation of the sponge circulatory system, we focused in the present study on comparable roles of extracellular matrix molecules.

In response to the extracellular adhesion molecule, galectin, the 3D-cell aggregates (primmorphs) from S. domuncula attach and start to arrange their cells in canal-like structures. The ability of S. domuncula to react to a homologous extracellular matrix by a morphogenetic effect as reported here and also recently by Adell et al. (Adell et al., 2003) and Wiens et al. (Wiens et al., 2003) is shared with the metazoan systems (Reed, 1990). The dramatic change in the organization pattern of the primmorphs, from spherical non-attached aggregates to flat more complex cellular entities is very reminiscent of mammalian spheroids induced to angiogenesis (Wartenberg et al., 2001). One of the evolutionary oldest angiogenesis-
controlling systems in Metazoa is CD36 and its ligand TSP (Calvo et al., 1995; Brower, 1999). No molecules related to the CD36 receptor or for the soluble TSP have been detected until now in non-metazoans. A comparison of CD36(-related) sequences from Metazoa, deposited in the database, with the sponge CD36/LIMPII receptor gives alignment scores ‘E’ (Expect value) (Coligan et al., 2000) of \(E^{-21}\); in contrast, the scores between the sponge molecule and the closest related molecules from yeast \(E=0.21\); Sp071p sequence) and plant \(E=3.1\); fructose bisphosphatase) show a more distant relationship. In the same way, the similarity between the \textit{S. domuncula} characterized by the expression of CD36/LIMPII receptor are located within the vessels, is inhibited by the angiogenesis-inhibitor TSP-1. The expression of the CD36/LIMPII receptor on the cell surface and thereby prevents the increased gene expression. This appears to be most likely in view of the existing data in other models and the competition experiment reported in the present study. Alternatively, the peptide may bind to another receptor that controls the expression of CD36/LIMPII, e.g. integrin, which had been identified in \textit{S. domuncula} (Wimmer et al., 1999). Therefore, binding studies of the recombinant sponge CD36/LIMPII receptor and the CSVTCG are in progress. It should be stressed that no other genes encoding a CD36- or CD36-related protein than that described here exist in the \textit{S. domuncula} EST library elaborated by us. Moreover, a screening with primers used in the present study and with other degenerate primers designed against CD36 did not result in the identification of a further, different cDNA for a putative CD36 receptor molecule in \textit{S. domuncula}.

Our results indicate that the canal formation in sponges is regulated by the tuned interaction between the CD36 receptor and the TSP-1 peptide. For a more direct proof of this assumption, inhibition studies with a secondary metabolite from a sponge were performed. MTN (2-methylthio-1,4-naphthoquinone) was isolated from a bacterial strain isolated from the sponge \textit{D. avara}. MTN was obtained by a bioassay-guided isolation. Our results now establish that MTN, previously known only as a synthetic product (Fieser and Brown, 1949) occurs also as a natural product. Along with other small naphthoquinone derivatives, MTN displays remarkable bioactivities: antifungal (Gershon and Shanks, 1975) and antitumor actions (Takano et al., 1960) of MTN had been described. The finding that MTN causes a strong antiangiogenic activity at very low doses is new and may qualify the compound for further therapy-oriented studies.

If MTN is added to primmorphs cultured on galectin the formation of the canal-like structures is prevented. The cells in such primmorphs remain in the spherical arrangement and no canals are seen. This effect occurs at a low concentration of \(0.03 \mu g/ml\). In parallel, the effect of MTN on the vessel formation in the chick chorio-allantoic membrane assay was determined. The alteration/prevention of the angiogenesis was achieved in the CAM assay at a dose of 1 ng/disc (5 pmol/disc). This potency is strong in comparison to other natural secondary metabolites, e.g. aeroplysinin (Rodrigues-Nieto et al., 2001), for which an anti-angiogenic activity was found at a dose of 14 nmol/disc. Subsequent cytotoxicity studies with mammalian tumor cells and MTN allowed an estimation of the cell growth inhibitory effect of this compound. In general, the cytotoxic activity of MTN was strong, but did not reach the level seen for the inhibition in the CAM assay. Using the murine leukemic lymphoblast cells L5178y, the threshold below which no cytotoxicity is seen is 30 ng/ml (150 nM). In comparison the cytotoxicity to rat adrenal pheochromocytoma PC-12 cells, or human cervix HeLa S3 cells, caused by MTN is even weaker; no effect on cell proliferation is seen below 300 ng/ml. Further investigations will include inhibition studies with endothelial mammalian cells.

We also investigated if MTN causes a modulating effect on the expression of the CD36 receptor (CD36/LIMPII receptor)-ligand (TSP) system in sponges. The expression of the CD36/LIMPII receptor gene is downregulated after incubation at a MTN concentration of 0.01 \(\mu g/ml\). Hence MTN shows the same vessel-inhibitory effect in both the vertebrate model and a the sponge system.

In summary, the major outcome of the present study is the finding that the CD36-thrombospondin system, hitherto known to be involved in tumor angiogenesis of vertebrates, might have an important role during formation of canals of the aquiferous system in sponges. This result has considerable impact on our understanding of the evolution of diseases in metazoans and the conservation of the pathways involved in development and host defense systems in multicellular animals. Recently, we already presented evidence that secondary metabolites produced by sponges or their associated microorganisms influence immune reactions, e.g. rejection of allogeneic tissue, in the same way as in vertebrates, including human. A striking example is myotrophin. In myocytes from mammals this protein stimulates protein synthesis (Sen et al., 1990), suggesting a crucial role in (reviewed by Sil et al., 1998). In the sponge system myotrophin has been demonstrated to likewise stimulate overall protein synthesis (Schröder et al., 2000).
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