Primmorphs generated from dissociated cells of the sponge *Suberites domuncula*: a model system for studies of cell proliferation and cell death

Marcio Reis Custodio [a,b], Ivo Prokic [a], Renate Steffen [a],
Claudia Koziol [a], Radovan Borojevic [b], Franz Brümmer [c],
Michael Nickel [c], Werner E.G. Müller [a,*]

[a] *Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität,
Duesbergweg 6, Mainz, D-55099, Germany*
[b] *Departamento de Histologia e Embriologia, Instituto de Ciêcias Biomédicas,
Universidade Federal de Rio de Janeiro, Rio de Janeiro, CEP 21941-970, CP 68021, Brazil*
[c] *Biologisches Institut, Abteilung Zoologie, Universität, Pfaffenwaldring 57, Stuttgart, 70550, Germany*

Abstract

Sponges (*Porifera*) represent the lowest metazoan phylum; they have been shown to be provided with the characteristic metazoan structural and functional molecules. One autapomorphic character of sponges is the presence of high levels of telomerase activity in all cells (or almost all cells, including somatic cells). In spite of this fact previous attempts to cultivate sponge cells remained unsuccessful. It was found that dissociated sponge cells do not replicate DNA and lose their telomerase activity. In addition, no nutrients or metabolites have been detected that would stimulate sponge cells to divide. In the present study we report the culture conditions required for the formation of multicellular aggregates from dissociated single cells of *Suberites domuncula*, termed primmorphs. These primmorphs are formed in seawater without addition of further supplements, and have an organised tissue-like structure; they have been cultured for more than 5 months. Cross-sections revealed a distinct

* Corresponding author. Tel.: +49 6131 395910; fax: +49 6131 395243; e-mail: wmueller@mail.uni-mainz.de

external layer covered by a continuous pinacoderm, and a central zone composed primarily of spherulous cells. After their association into primmorphs, the cells turn from the telomerase-negative state into the telomerase-positive state; a telomerase level of 4.7 total product generated (TPG) units/5×10^3 cell equivalents has been determined. Moreover, a major fraction of the cells in the primmorphs undergoes DNA synthesis and hence has the capacity to grow. Applying the BrdU-labelling and detection assay it is demonstrated that up to 33.8% of the cells in the primmorphs are labelled with BrdU after an incubation period of 12 h. It is proposed that the primmorph system described here is a powerful novel model system to study basic mechanisms of cell proliferation and cell interaction, as well as of morphogenesis, ageing and apoptosis. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

1. Introduction

Both palaeontological and comparative cell-biological studies indicate that the phylum *Porifera* (sponges) is a very ancient one and potentially the most simple among eumetazoans. Recent molecular studies have given substantive support to the hypothesis that *Porifera* and other animals belong to a single monophyletic kingdom (reviewed in Müller, 1995). *Porifera* have been extensively studied in recent years, in order to establish cellular and molecular aspects of the evolution from unicellular to multicellular grade of organisation in animals.

The full cell function of multicellular organisms requires both structural integration and molecular communication, and one of the key autapomorphic characters of metazoa is the receptor tyrosine kinase signalling pathway, which is found only in this kingdom, including sponges (Müller and Schäcke, 1996). In view of the other signalling pathways that are also present in sponges, such as retinoic acid, inositol phosphates, or protein kinase C (Rottmann et al., 1987; Biesalski et al., 1992; Imsiecke et al., 1994), it has been assumed that sponge cells follow the general pattern of integration and communication observed in higher metazoans. In contrast, *Porifera* comprise a character that is not found in higher metazoan phyla: they have high levels of telomerase activity in normal tissues (Koziol et al., 1998). This fact implies that sponges do not show a clear distinction between the germ- and somatic-cell lineages (reviewed in Müller, 1998a,b). In higher metazoa, germ cells and early embryonic cells are telomerase-positive while somatic cells are telomerase-negative: a brief transitory induction of telomerase activity can be observed in tissue stem cells involved in intense tissue repair and regeneration, but the permanent telomerase activity is associated only with the conversion of normal cells into malignant neoplastic cells (Lange, 1998).

Most sponges are long-lived, and some reach a life span of more than 1500 years (Lehnert and Reitner, 1997). This observation fits well with the fact that most cells

from sponges are telomerase-positive (Koziol et al., 1998). Therefore, it might be inferred that sponge cells have an unlimited capacity for proliferation associated with their differentiation into normal tissue cells during their growth and regeneration. In agreement, until now there are no reports on neoplastic diseases in sponges (De-Flora et al., 1995). However, in the initial report on the presence of high levels of telomerase in sponge cells, it was shown that cells that have lost contact with each other become telomerase-negative (Koziol et al., 1998). Cells kept in a single cell suspension either die, very likely in a process of apoptosis (Wagner et al., 1998), or remain in a non-proliferating dormant state, in agreement with the observed correlation between proliferation and telomerase activity in cells of higher organisms (Belair et al., 1997).

The high proliferation capacity of sponge cells suggests that it should be easily feasible to establish their cell cultures in vitro. However, until now, only the maintenance of sponge cells in vitro has been achieved, e.g. from *Hymeniacidon heliophila* (Pomponi and Willoughby, 1994), *Ephydatia muelleri* (Imsiecke et al., 1995b), *Latrunculia magnifica* (Ilan et al., 1996) and *Suberites domuncula* (Müller and Schäcke, 1996). The cells in suspension did not proliferate readily (Ilan et al., 1996) and the proposed experimental systems cannot be termed primary cell cultures (Freshney, 1994). In addition, primary single cell suspensions from sponges also contain bacteria and protozoa as shown by earlier experiments (Klautau et al., 1994; Custodio et al., 1995). The reasons for the fact that the cells remained in the resting stage might be found (i) in the experimental approach to establish a single cell culture, and (ii) in the culture conditions used. The media used were supplemented with fetal bovine serum (Pomponi and Willoughby, 1994; Ilan et al., 1996) under the assumption that the growth factors present in the serum might stimulate cell proliferation. However, it appears reasonable to accept that sponge cell surface receptors are not activated by ligands present in bovine serum. Furthermore, serum-rich media imply a risk of protozoan contamination (Osinga et al., 1998).

It has been known for a long time that dissociated sponge cells reaggregate and potentially reorganise into a fully functional and structured sponge (Wilson, 1907), and this experimental model has been extensively used to study sponge cell differentiation and morphogenesis (Curtis, 1962; Borojevic and Lévi, 1964). The dissociated cells are either induced to death and phagocyted by archaeocytes, or reaggregate and subsequently sort out following adhesion and motility gradients, described as contact-promoting by Curtis (1962). This leads to the formation of a compact spherical body, contrasting with rather loose irregular cellular contacts during the aggregation (Müller, 1982). The formation of a pinacoderm, either from pre-existing pinacocytes or from archaeocytes (reviewed in Borojevic, 1970, 1971), represents the first step in reorganisation of tissue-like structures. This stage, termed here 'primmorphs', represents the end of the aggregation of cellular material and the separation of the internal milieu from the external environment by a continuous pinacoderm. Under favourable conditions, associated in general with adherence and stable fixation onto the solid substrate, this stage will lead to morphogenetic processes ending in the full reorganisation of the sponge body (to be published). The primmorphs thus represent new structures obtained from single cells, and

resemble partially a broader concept of diamorphs, defined by Borojevic et al. (1967) as the stage that is simultaneously the end of the concentration processes (such as observed in reduction bodies, external buds, and internal gemmules) and the beginning of the sponge regeneration and expansion (Borojevic, 1970, 1971).

In view of the observation that telomerase can be reactivated after reestablishment of cell to cell contacts, and that the cell viability can be retained for long periods in primmorphs, we have questioned whether the cells actively proliferate and whether the telomerase activity was fully established. The simultaneous reestablishment and preservation of cell to cell and cell to matrix contacts, as well as of the internal milieu and the potential maintenance of symbiotic micro-organisms which may be required for the full sponge cell viability, indicates the culture of primmorphs as a promising experimental system for sponge cell culture ex vivo and for studies of molecular processes of development and ageing in representatives of the ancient metazoans, the *Porifera*. Instead of stimulating the primmorph attachment and conversion into the normal and functional sponge, we have followed up the long-term cell viability in unattached primmorphs.

2. Materials and methods

2.1. Materials

Natural seawater (S9148), penicillin and streptomycin were obtained from Sigma (Deisenhofen, Germany), RNAguard (24 000 U/ml) from Pharmacia (Freiburg, Germany), the Telomerase Detection Kit (TRAPeze) from Oncor (Gaithersburg, MD), BrdU-labelling and detection kit (cat. no. 1299964) from Boehringer Mannheim (Mannheim, Germany), and SYBR Green I from Molecular Probes (Leiden, Netherlands).

The compositions of Ca^{2+} and Mg^{2+}-free artificial seawater (CMFSW) as well of CMFSW containing EDTA (CMFSW-E) were described previously (Rottmann et al., 1987).

2.2. Sponge

Specimens of the marine sponges *S. domuncula* (*Porifera*, *Demospongiae*, *Hadromerida*) were collected in the Northern Adriatic near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) at a temperature of 16°C.

2.3. Dissociation of cells and formation of primmorphs

All cell culture dishes and tubes were sterilised and the media were filtrated through 0.2-μm polycarbonate filters. Tissue samples of 4–5 cm³ were submersed in seawater in Petri dishes and cut into 1-mm³ cubes; they were transferred into 50-ml conical tubes (Falcon no. 2070) filled with CMFSW-E (ratio tissue to medium 1 : 10). After gentle shaking for 30 min at 16°C with a rotatory shaker, the solution

was discarded and new CMFSW-E was added. After 40 min the supernatant was collected and filtered through 40-μm mesh nylon net; the process of shaking in CMFSW-E (40 min) and filtration was repeated once again. The single cells were harvested by centrifugation (500 $\times$ g for 5 min) and washed once in CMFSW. The cells of the second pellet were resuspended in seawater supplemented with antibiotics (100 IE of penicillin and 100 μg/ml of streptomycin; seawater/antibiotics). A cell suspension of 10^7 cells was added to 6 ml (final volume) of seawater/antibiotics in 60-mm Petri dishes (Falcon no. 3004).

Each day two-thirds of the culture medium was replaced by fresh seawater/antibiotics; the cell clumps formed were resuspended to avoid adhesion of cells to the plate. Cell aggregates of a diameter of at least 0.5 mm were collected by gentle pipetting as soon as they had formed (and using gravity only for the separation from smaller aggregates and from single cells); they were washed twice with 10 ml of seawater. The suspension of cells and aggregates was transferred into 15-ml tubes (Falcon no. 2096) filled with 12 ml of seawater. This collection of aggregates from the Petri dishes was repeated after a further day of incubation. The aggregates were transferred again into new Petri dishes (total volume of 6 ml). During this procedure foreign organisms, mainly protozoa which attached to the dishes, were removed from the sponge cell aggregates which remained in the suspension.

The round-shaped primmorphs, were placed into 24-well plates (Nunclon™ no. 143982) (one–two/well) and 1 ml of seawater/antibiotics was added. Medium was changed every day during the first 2 weeks; later the medium change was necessary only once or twice a week. All pipettings were performed by the aid of Pasteur pipettes (diameter of the openings: 2 mm) or plastic tips (diameter: 2–3 mm). Where indicated, the primmorphs were dissociated with CMFSW-E.

2.4. Incubation of primmorphs with BrdU: immunocytochemical detection of DNA synthesis

For the determination of cell proliferation the incorporation of BrdU (5-bromo-2′-deoxy-uridine) into cellular DNA was monitored using the BrdU-labelling and detection kit as recommended by the manufacturer. Primmorphs were incubated in 1 ml seawater/antibiotics, supplemented with BrdU-labelling medium (final dilution of 1 : 1000; 10 μM of BrdU), for 12 h using culture chambers (culture chamber slides, Nunc no. 177453). The cells were dissociated in CMFSW-E, washed three times in CMFSW-E and fixed in 70% ethanol (pH 2.0, acetate buffer). The cells were incubated with anti-BrdU mouse monoclonal antibody. After 30 min (37°C) the cells were incubated with anti-mouse Ig-alkaline phosphatase, and the immunocomplexes were visualised with the colour-substrate nitroblue tetrazolium salt. The cells were observed and scored under a light microscope, using an Olympus AHBT3 microscope.

2.5. Histological analysis

Primmorphs were fixed in 4% paraformaldehyde/PBS (Romeis, 1989). After dehydration in ethanol, the samples were embedded in Technovit 8100 (Beckstead, 1985), according to the instructions of the manufacturer. Sections of 2-μm thickness were prepared and stained with Ziehl's fuchsin (Martoja and Martoja, 1967).

2.6. Telomerase assay

Telomerase activity was determined by polymerase chain reaction (PCR) procedure applying the 'Telomerase Detection Kit (TRAPeze)' as described (Kim et al., 1994; Koziol et al., 1998). The cell extracts corresponded to 5×10^3 cell equivalents. The amplification products were separated by electrophoresis through a 12.5% non-denaturing polyacrylamide gel in $0.5 \times$ TBE-buffer according to the instructions of the supplier. The gels were stained with SYBR Green I to detect the DNA fragments (Molecular Probes, 1996). The signals were quantified by application of a GS-525 Molecular Imager (Bio-Rad). The amount of telomerase activity is given in units of TPG (total product generated) and was calculated as described (Oncor, 1996).

The number of cells analysed was estimated on the basis of the protein and DNA content of the extract to be assayed. Based on the DNA content per cell (3.7 pg/cell for *S. domuncula*; Imsiecke et al., 1995a), the number of cell equivalents was calculated for a given tissue extract.

3. Results

3.1. Formation of primmorphs from cells of S. domuncula

The cells were obtained from specimens of the sponge *S. domuncula* (Fig. 1A). Single cell suspensions, obtained by dissociation in the absence of Ca^{2+} and Mg^{2+}, were plated onto plastic dishes. Non-adherent sponge cells were harvested and washed twice to remove most protozoa, which preferentially adhered to the plastic culture dishes. Thereafter, the cells were maintained in seawater/antibiotics. The cell aggregates steadily increased in size until the third day, through fusion or incorporation of single cells (Fig. 1B and C). After a total treatment/incubation period of 5 days, typical round-shaped primmorphs were formed whose size ranged from 1 to 2 mm (Fig. 1D).

The outer appearance of the primmorphs was smooth and nearly spherical (Fig. 1D). Microscopic analysis of the primmorph sections stained with Ziehl's fuchsin revealed that the cells were well organised in distinct tissue-like regions (Fig. 1E and F). A pronounced layer of epithelium-like cells (Fig. 1E and F) surrounded the central mass of cells. The cells that composed this external layer of the primmorphs were identified as pinacocytes, following their flattened form and prominent nuclei (Simpson, 1984); their size ranged from 15 to 20 μm. Several layers of amoeboid

cells were found in this region, subjacent to the pinacoderm. The cells in the central part of primmorphs were mostly spherulous cells. Their diameter was 40–45 μm and they were characterised by large round vacuoles that occupied most of the cell bodies. Other cells corresponded to archaeocytes (Bergquist, 1978) and had a size ranging from 55 to 60 μm. Bacteria were frequently present inside the primmorphs of *S. domuncula*, delimited from the sponge tissue inside the vacuoles of a specific cell type (to be published).

The organised arrangement of the cells within the primmorphs distinguishes them from primary cell aggregates which are formed from dissociated cells in the presence of the homologous aggregation factor (Müller, 1982).

3.2. Subcultures of primmorphs

The primmorphs were kept in culture in the seawater/antibiotics medium. Under those conditions they could be kept for over 5 months in a viable state.
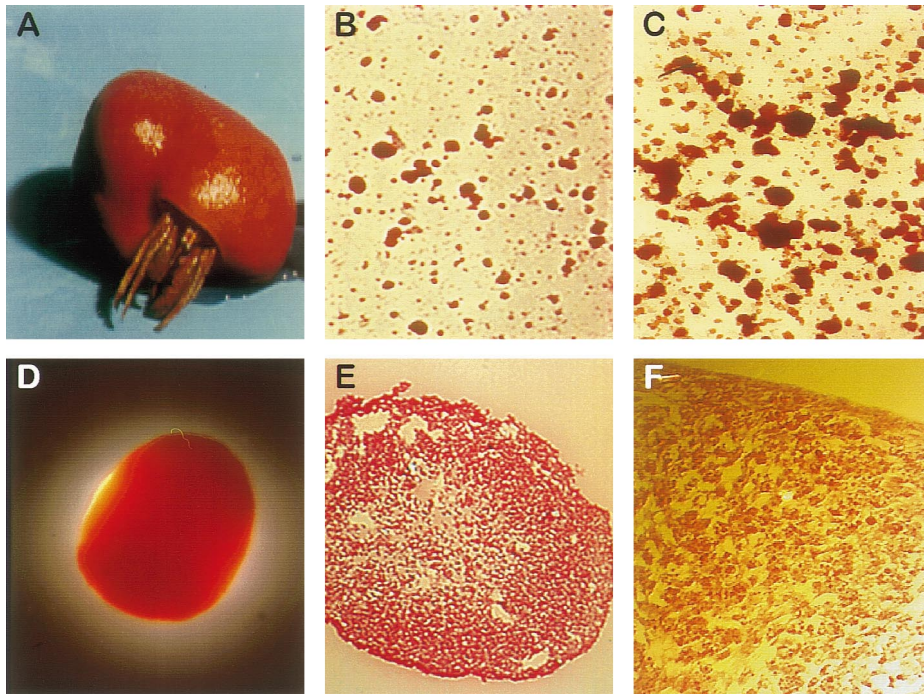

Fig. 1. Formation of primmorphs from cells of the sponge *S. domuncula*. (A) A specimen of *S. domuncula*; magnification $\times 1$. (B) Cells were dissociated in CMFSW-E. Cell aggregates are formed after 1 day in culture (medium: seawater/antibiotics) ($\times 10$), which (C) increase in size (micrograph taken after 3 days) ($\times 10$), and (D) 5 days after incubation primmorphs are formed ($\times 15$). (E, F) Cross-sections through a primmorph, showing the single-cellular layer of epithelial-like pinacocyte surrounding the internal part, composed of spherulous cells and archaeocytes including amoebocytes; E, $\times 30$; F, $\times 60$.
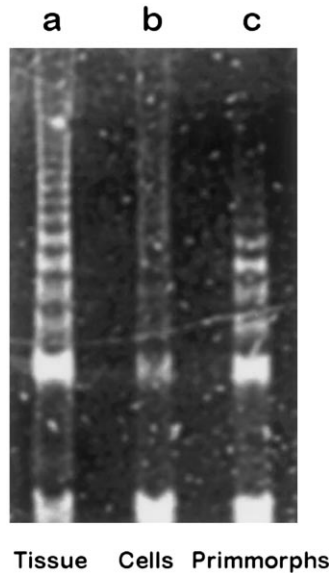
Fig. 2. Telomerase activity in cells from *S. domuncula*. Telomerase activity was determined in cells present (i) in tissue (lane a), (ii) in the dissociated single-cell state (the cells have been analyzed 14 h after dissociation) (lane b), and (iii) in primmorphs (lane c). Defined amounts of tissue, corresponding to 5×10^3 cell equivalents, were assayed in the TRAP assay. After PCR amplification the products were resolved in a non-denaturing polyacrylamide gel and the gels were stained with SYBR Green I to detect the DNA fragments.

Either immediately after their formation or after a long-term culture, the primmorphs can be dissociated again using CMFSW-E. When transferred into seawater/antibiotics, single cell suspensions formed aggregates and subsequently small primmorphs, which were termed secondary primmorphs. The kinetics of their formation was identical to that seen for the primary primmorphs. In the absence of Ca^{2+}, using the medium CMFSW, the single cells obtained after dissociation of primmorphs readily attached to the surface of glass dishes. For optimal attachment to the plastic surface, the plastic dishes had to be scratched moderately using the tip of a pipette or a plastic rubber (not shown).

3.3. Level of telomerase activity in sponge cells depending upon the culture conditions

As previously reported, sponge cells undergo a transition from the telomerase-positive to a telomerase-negative state after dissociation into a single-cell suspension (Koziol et al., 1998).

The level of telomerase activity was determined in cells during formation of primmorphs from a single-cell suspension. Sponge cells contain high levels of telomerase activity in their natural tissue location; a quantitative analysis revealed an activity of 8.9 TPG units/5×10^3 cell equivalents (Fig. 2, lane a). When the

telomerase activity was determined in cells that had been left for 14 h in the dissociated single-cell state, the enzyme level dropped to 0.9 TPG units/5×10^3 cells (Fig. 2, lane b). However, when cells from primmorphs (10 days after formation from single cells) were used for the analysis, a telomerase activity of 4.7 TPG units/5×10^3 cells was observed (Fig. 2, lane c). These data confirm that cells lose their telomerase activity when removed from tissues. As postulated earlier, single cells will recover after formation of tissue-like organisation into primmorphs, and turn from the telomerase-negative to the telomerase-positive state (Koziol et al., 1998; Wagner et al., 1998).

3.4. Immunocytochemical detection of BrdU incorporation in cells of primmorphs

The BrdU-labelling and detection assay (Gratzner, 1982) was used to demonstrate that the cells organised into the primmorphs regain the capacity to proliferate. As a measure of proliferation, the cells were incubated for 12 h in the presence of BrdU. The incorporation of BrdU into DNA was detected by using an anti-BrdU monoclonal antibody as described in Section 2.

The BrdU-positive cells undergoing DNA synthesis stained brown in their nuclei (Fig. 3B–D). In a control assay, the antibody against BrdU was omitted, and under this condition no staining is observed (Fig. 3A).
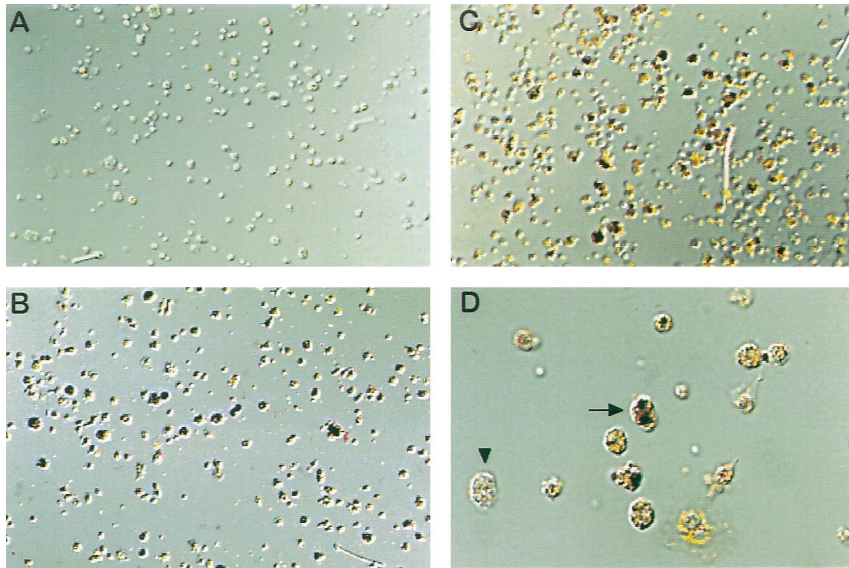

Fig. 3. Immunocytochemical detection of proliferating (BrdU-labelled) cells from primmorphs of *S. domuncula*. After incubation of the primmorphs with BrdU the cells were dissociated and subjected to staining with anti-BrdU monoclonal antibody as described in Section 2. The dark brownish stained nuclei are those which incorporated BrdU (B–D); in D the arrow marks a BrdU-positive cell and the arrow head a BrdU-negative cell. A shows a control in which the antibody against BrdU was omitted. A and B, magnification ×20; C, ×40; D, ×125.

Table 1
Analysis of cells for DNA synthesis applying the BrdU-labelling and detection assay

Cells analyzed	Percentage of BrdU-positive cells (%)
Dissociated cells (after 24 h)	0
Aggregates (after 24 h)	6.5
Primary primmorphs (3 days)	33.8
Primary primmorphs (1 month)	22.3

A single cell suspension was incubated with BrdU and the incorporated nucleotides were visualized immunologically using anti-BrdU monoclonal antibody. The percentage of BrdU-positive cells is given. The analysis was performed with (i) dissociated cells which had been kept for 1 day in CMFSW-E, (ii) cell aggregates formed from single cells after 1 day in culture with seawater, (iii) primary primmorphs after formation for 3 days and (iv) primary primmorphs after 1 month in culture. Per assay 300 cells were counted.

Suspensions of dissociated cells, which had been kept for 1 day in CMFSW showed no cells with active DNA synthesis (Table 1). The percentage of BrdU-positive cells in cell aggregates formed from single cells after 1 day in culture was low, reaching only 6.5%. In contrast, the number of DNA-synthesising and proliferating cells present in primmorphs was high. As summarised in Table 1, the number of BrdU-positive cells in primary primmorphs was 33.8% and in 'older' primmorphs, after 1 month in culture, 22.3%. These data document that cells incorporated and reorganised into tissue-like primmorphs undergo DNA synthesis and subsequent cell division.

4. Discussion

The major objective of the present study was the development of experimental conditions that support a long-term sponge cell culture in vitro, in order to study controls of cell proliferation and differentiation. As outlined in Section 1, so far the sponge cells were only maintained in vitro for a limited period of time. In experiments in our laboratory until now we also failed to compose a culture medium for single cells in suspension culture.

In a rational approach we could demonstrate that after dissociation the sponge cells lose the telomerase activity and in consequence their intriguing property to proliferate and to constitute (to a large extent) immortal cell populations (Koziol et al., 1998; Wagner et al., 1998Fig. 4). In addition, in a series of trials we failed to identify supplements for the culture medium supplying the growth factors known to be required for invertebrate and lower vertebrate cells to grow (data not shown). Based on these facts, we postulated that for an efficient culture of dividing sponge cells, the following prerequisites are necessary: (i) viable cells, (ii) provision of cell-cell and/or cell-matrix contacts, and (iii) suitable nutrients presented in a form that can be used by sponge cells. These criteria have been met with the establishment of primmorph cultures.

In order to obtain viable cells, we have used dissociation procedures based on the elimination of bivalent cations, shown in previous studies to mediate sponge cells adhesion (reviewed in Müller et al., 1988). This method could be applied with success both to primary sponge tissues and to primmorphs.

Sponge cells live inside or on an organic structured matrix (Simpson, 1984) and are expected to be anchorage-dependent. Simultaneously, the attachment onto a solid substrate of sponge cell diamorphs, external buds or gemmules has been shown to be strictly required for the sponge tissue reorganisation and morphogenesis (reviewed in Borojevic, 1971). Accordingly, previous attempts to establish isolated sponge cell cultures have used artificial solid substrates, associated or not with biogenous molecules such as collagen, in order to provide the required conditions for anchorage of sponge cells in vitro. In the present study, besides the mechanical support for cell attachment, the culture model using primmorphs establishes the cell-cell contacts as well as the cell contacts with the endogenous extracellular matrix. The presence of specific collagen types, a major structural element required for cell adhesion and migration, has been thoroughly documented in sponges (Garrone, 1985). The existence of the adhesive glycoprotein fibronectin, known from higher invertebrates and vertebrates to mediate cellular interactions with the extracellular matrix (Hynes, 1990), has been proven—until now—only by immune cross-reactivity with heterologous antibodies, and by isolation of a putative cDNA encoding this polypeptide from *G. cydonium* (Pahler et al., 1998a). The presence of morphogens in primmorphs has to be postulated, in order to understand the precise arrangement of proliferating cells in these bodies. Recently a
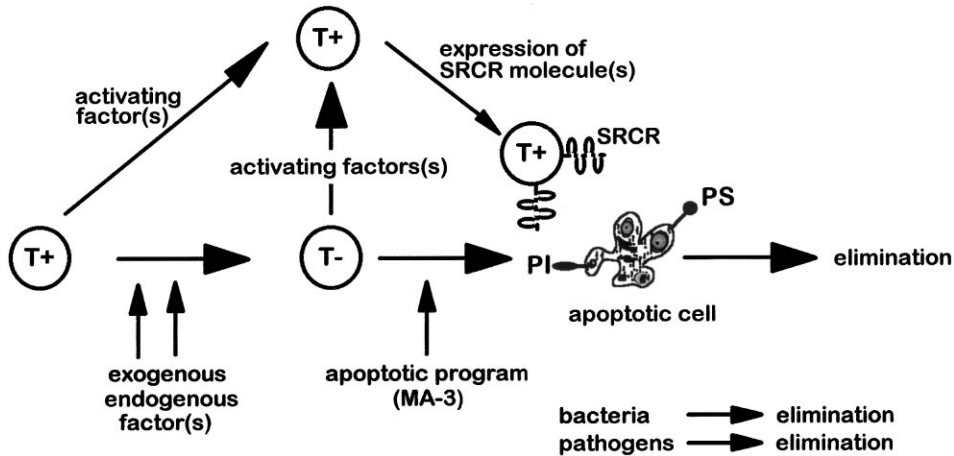

Fig. 4. Proposed scheme for the explanation of the transition of telomerase-positive- (T$^+$) to telomerase-negative cells (T$^-$) with the consequence of apoptotic cell death. It is outlined that exogenous and/or endogenous factor(s), cause the transition from T$^+$ − to T$^-$-cells; during this process the apoptotic program is initiated. The apoptotic cells expose both phosphatidyserine (PS) and phosphatidylinositol (PI); these phospholipids bind to T$^+$-cells which express on their surface receptors composed of scavenger receptor cysteine-rich repeats (SRCR). Further details are given in Section 4.

cDNA, encoding the potential morphogen endothelial-monocyte-activating polypeptide, has been also isolated from the sponge *G. cydonium* (Pahler et al., 1998b). The proposed model of in vitro culture should open the possibility to study the biological activity of such molecules.

The sponge cells assembled in primmorphs become telomerase-positive and show DNA synthesis and hence regain the prerequisites for cell growth. One cause for this transition must be seen in the fact that the cells have recovered the physiological contact to the neighbouring cells and/or to the homologous extracellular matrix and in consequence have arranged a functional organisation. This is similar to cultures of embryoid bodies from mammalian embryonic stem cells, which provide the conditions for both proliferation and differentiation of totipotent and telomerase positive early embryo cells (Keller, 1995). Three-dimensional cell cultures are frequently required for experimental cell differentiation models of higher animals, but not for cell proliferation (Müller-Klieser, 1997), and the fact that sponge cells require this mode of culture for proliferation remains to be explained.

Sponge nutritional physiology is poorly known. Most studies have shown that phagocytosis is probably the major input pathway for nutritional materials into sponges (Willenz et al., 1986; Van de Vyver et al., 1990), although the presence of specific cell categories that store glycogen and lipids would suggest internal circulation of energy-transport molecules such as glucose (Boury-Esnault, 1977). It is well established that archeocytes, choanocytes or spherulous cells are active in phagocytosis of cells and debris (Simpson, 1984), and this finding is supported also by enzymatic data (Krasko et al., 1997). Phagocytosis of cell debris and cells undergoing apoptosis is mediated by receptors, including scavenger receptors with cysteine-rich repeats (Krieger and Herz, 1994; Fig. 4). Receptors belonging to this family have been identified in sponges (Pancer et al., 1997; Pahler et al., 1998a). At present, no experimental data are available on factors which might be involved in the expression of these receptors.

Knowing from earlier studies that sponges live in a symbiotic and commensalic relationship with bacteria (Müller et al., 1981; Althoff et al., 1998) and/or algae (Vacelet, 1971; Gilbert and Allen, 1973) it was reasonable to develop a procedure by which sponge cells retain the ability to maintain the symbiotic relationship to bacteria and—if present—also to algae. Therefore, the cells were dissociated and allowed to reaggreate under conditions which facilitate reaggregation of sponge cells with their potential symbionts.

Finally, similar to the ability of sponges to extrude debris or unused spicules, the primmorphs extruded cell debris observed to be deposited at the bottom, around the free unattached primmorphs, and this phenomenon may be relevant for the maintenance of the cell viability in primmorphs.

In conclusion, it can be suggested that both apoptotic cells and bacteria or other organisms are eliminated via binding to scavenger receptors and serve as suitable nutrients for the support of the cell metabolism. In addition, sponge cells apparently require cell-cell contacts for DNA synthesis and growth. The primmorph system described here can be considered to be a powerful novel model to study basic mechanisms of cell proliferation and cell death; it can be applied e.g. to the

analysis of molecular events causing the transition from telomerase-positive to telomerase-negative cells. In addition, this system may be used in the future as bioreactor to produce bioactive compounds from sponges.

Acknowledgements

References

Althoff, K., Schütt, C., Krasko, A., Steffen, R., Batel, R., Müller, W.E.G., 1998. Evidence for a symbiosis between bacteria of the genus *Rhodobacter* and the marine sponge *Halichondria panicea*: harbor also for putatively-toxic bacteria? Mar. Biol. 130, 529–536.

Beckstead, J.H., 1985. Optimal antigen localization in human tissues using aldehydefixed plastic-embedded sections. J. Histochem. Cytochem. 9, 954–958.

Belair, C.D., Yeager, T.R., Lopez, P.M., Reznikoff, C.A., 1997. Telomerase activity: a biomarker of cell proliferation, not malignant transformation. Proc. Natl. Acad. Sci. USA 94, 13677–13682.

Bergquist, P.L., 1978. Sponges. Hutchinson, London, 268 pp.

Biesalski, H.K., Doepner, G., Tzimas, G., Gamulin, V., Schröder, H.C., Batel, R., Nau, H., Müller, W.E.G., 1992. Modulation of *myb* gene expression in sponges by retinoic acid. Oncogene 7, 1765–1774.

Borojevic, R., 1970. Differenciation cellulaire dans l'embryogenèse et morphogenèse ches les spongiaires. In: Fry, W.G. (Ed.), The Biology of *Porifera*. Academic Press, London, pp. 467–490.

Borojevic, R., 1971. Le comportement des cellules d'éponges lors des processus morphogénétiques. Ann. Biol. 10, 533–539.

Borojevic, R., Lévi, C., 1964. Etude au microscope électronique des cellules de l'éponge *Ophlitaspongia serriata* (Grant) au cours de la réorganisation après dissociation. Z. Zellforsch. Mikroskop. Anat. 64, 708–725.

Borojevic, R., Fry, W.G., Jones, W.C., Lévi, C., Rasmont, R., Sarà, M., Vacelet, J., 1967. A reassessment of the terminology for sponges. Bull. Mus. Natl. Hist. Nat. Paris 39, 1225–1235.

Boury-Esnault, N., 1977. A cell type in sponge involved in the metabolism of glycogen: the gray cells. Cell Tissue Res. 175, 523–539.

Curtis, A.S.G., 1962. Pattern and mechanism in the reaggregation of sponges. Nature 196, 245–248.

Custodio, M.R., Imsiecke, G., Borojevic, R., Rinkevich, B., Rogerson, A., Müller, W.E.G., 1995. Evolution of cell adhesion systems: evidence for Arg-Gly-Asp mediated adhesion in the protozoan *Neoparamoeba aestuarina*. J. Protozool. Eukary. Res. 42, 721–724.

De-Flora, S., Bagnasco, M., Bennicelli, C., Camoirano, A., Bojnemirski, A., Kurelec, B., 1995. Biotransformation of genotoxic agents in marine sponges. Mechanisms and modulation. Mutagenesis 10, 357–364.

Freshney, R.I., 1994. Culture of Animal Cells, 3rd ed. Wiley-Liss, New York, 486 pp.

Garrone, R., 1985. The collagen of the *Porifera*. In: Bairati, A., Garrone, R. (Eds.), Biology of Invertebrate and Lower Vertebrate Collagens. Plenum, London, pp. 157–175.

Gilbert, J.J., Allen, H.L., 1973. Chlorophyll and primary productivity of some green, freshwater sponges. Int. Rev. Ges. Hydrobiol. 58, 633–658.

Gratzner, H.G., 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 218, 474–475.

Hynes, R.P., 1990. Fibronectins. Springer, New York.

Ilan, M., Contini, H., Carmeli, S., Rinkevich, B., 1996. Progress towards cell cultures from marine sponges that produce bioactive compounds. J. Mar. Biotechnol. 4, 145–149.

Imsiecke, G., Borojevic, R., Müller, W.E.G., 1994. Retinoic acid acts as a morphogen in freshwater sponges. Invertebr. Reproduc. Dev. 26, 89–98.

Imsiecke, G., Custodio, M., Borojevic, R., Steffen, R., Moustafa, M.A., Müller, W.E.G., 1995a. Genome size and chromosomes in marine sponges (*Suberites domuncula*, *Geodia cydonium*). Cell Biol. Int. 19, 995–1000.

Imsiecke, G., Steffen, R., Custodio, M.R., Borojevic, R., Müller, W.E.G., 1995b. Formation of spicules by sclerocytes from the freshwater sponge *Ephydatia muelleri* in short-term cultures in vitro. In Vitro Cell. Dev. Biol. 31, 528–535.

Keller, G.M., 1995. In vitro differentiation of embryonic stem cells. Curr. Opin. Cell Biol. 7, 862–869.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E., Weinrich, S.L., Shay, J.W., 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266, 2011–2014.

Klautau, M., Custodio, M.R., Borojevic, R., 1994. In vitro culture of primary cell lines from marine sponges. In: van Soest, R., Balkema, A.A. (Eds.), Sponges in Time and Space. Brookfield, Rotterdam, pp. 401–406.

Koziol, C., Borojevic, R., Steffen, R., Müller, W.E.G., 1998. Sponges (*Porifera*) model systems to study the shift from immortal to senescent somatic cells: the telomerase activity in somatic cells. Mech. Ageing Dev. 100, 107–120.

Krasko, A., Gamulin, V., Seack, J., Steffen, R., Schröder, H.C., Müller, W.E.G., 1997. Cathepsin, a major protease of the marine sponge *Geodia cydonium*: purification of the enzyme and molecular cloning of cDNA. Mol. Mar. Biol. Biotechnol. 6, 296–307.

Krieger, M., Herz, J., 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). Annu. Rev. Biochem. 63, 601–637.

Lange, T.V., 1998. Telomeres and senescence: ending the debate. Science 279, 334–335.

Lehnert, H., Reitner, J., 1997. Lebensdauer und Regeneration bei *Ceratoporella nicholsoni* (Hickson, 1911) and *Spirastrella* (Acanthochaetetes) *wellsi* (Hartman and Goreau, 1975). Geol. Bl. NO-Bayern 47, 265–272.

Martoja, R., Martoja, M., 1967. Initiation aux Techniques de l'Histologie Animale, 1st ed. Masson, Paris.

Molecular Probes, 1996. Product information sheet: SYBR Green I nucleic acid gel stain, MP 7567 07/16/96. Molecular Probes, Leiden, Netherlands.

Müller, W.E.G., 1982. Cell membranes in sponges. Int. Rev. Cytol. 77, 129–181.

Müller, W.E.G., 1998a. Molecular phylogeny of eumetazoa: experimental evidence for monophyly of animals based on genes in sponges (*Porifera*). Prog. Mol. Subcell. Biol. 19, 98–132.

Müller, W.E.G., 1998b. Origin of metazoa: sponges as living fossils. Naturwissenschaften 85, 11–25.

Müller, W.E.G., 1995. Molecular phylogeny of metazoa (animals): monophyletic origin. Naturwissenschaften 82, 321–329.

Müller, W.E.G., Schäcke, H., 1996. Characterization of the receptor protein-tyrosine kinase gene from the marine sponge *Geodia cydonium*. Prog. Mol. Subcell. Biol. 17, 183–208.

Müller, W.E.G., Zahn, R.K., Kurelec, B., Lucu, C., Müller, I., Uhlenbruck, G., 1981. Lectin, a possible basis for symbiosis between bacteria and sponges. J. Bacteriol. 145, 548–558.

Müller, W.E.G., Diehl-Seifert, B., Gramzow, M., Friese, U., Renneisen, K., Schröder, H.C., 1988. Interrelation between extracellular adhesion proteins and extracellular matrix in reaggregation of dissociated sponge cells. Int. Rev. Cytol. 111, 211–229.

Müller-Klieser, W., 1997. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. Am. J. Physiol. 273, C1109–1123.

Oncor, 1996. TRAPeze telomerase detection kit, catalog no. S7700-Kit, 2nd ed. Oncor, Gaithersburg, MD.

Osinga, R., Tramper, J., Wijffels, R.H., 1998. Cultivation of marine sponges for metabolite production: application for biotechnology? Trends Biotechnol. 16, 130–134.

Pahler, S., Blumbach, B., Müller, I.M., Müller, W.E.G., 1998a. A putative multiadhesive basal lamina protein from the marine sponge *Geodia cydonium*: cloning of the cDNA encoding a fibronectin-, an SRCR- as well as a complement control protein module. J. Exp. Zool. (in press).

Pahler, S., Krasko, A., Schütze, J., Müller, I.M., Müller, W.E.G., 1998b. Isolation and characterization of a cDNA encoding a potential morphogen from the marine sponge *Geodia cydonium*, that is conserved in higher metazoans. Proc. R. Soc. London B. 265, 421–425.

Pancer, Z., Münkner, J., Müller, I., Müller, W.E.G., 1997. A novel member of an ancient superfamily: sponge (*Geodia cydonium, Porifera*) putative protein that features scavenger receptor cysteine-rich repeats. Gene 193, 211–218.

Pomponi, S.A., Willoughby, R., 1994. Sponge cell culture for production of bioactive metabolites. In: van Soest, R., Balkema, A.A. (Eds.), Sponges in Time and Space. Brookfield, Rotterdam, pp. 395–400.

Romeis, B., 1989. Mikroskopische Technik. Urban und Schwarzenberg, München.

Rottmann, M., Schröder, H.C., Gramzow, M., Renneisen, K., Kurelec, B., Dorn, A., Friese, U., Müller, W.E.G., 1987. Specific phosphorylation of proteins in pore complex-laminae from the sponge *Geodia cydonium* by the homologous aggregation factor and phorbol ester. Role of protein kinase C in the phosphorylation of DNA topoisomerase II. EMBO J. 6, 3939–3944.

Simpson, T.L., 1984. The Cell Biology of Sponges. Springer, New York.

Vacelet, J., 1971. Etude en microcopie électronique de l'association entre une Cyanophycée Chroococcale et une éponge du genre *Verongia*. J. Microsc. 12, 363–380.

Van de Vyver, G., Vray, B., Belauane, S., Toussaint, D., 1990. Efficiency and selectivity of microorganism retention by *Ephydatia fluviatilis*. In: Rützler, K., Macintyre, V.V., Smith, K.P. (Eds.), New Perspectives in Sponge Biology. Smithsonian Institution Press, Washington, DC, pp. 511–515.

Wagner, C., Steffen, R., Koziol, C., Batel, R., Lacorn, M., Steinhart, H., Simat, T., Müller, W.E.G., 1998. Apoptosis in marine sponges: a biomarker for environmental stress (cadmium and bacteria). Mar. Biol. 131, 411–421.

Willenz, P., Vray, B., Maillard, M.P., Van de Vyver, G., 1986. A quantitative study of the retention of radioactively labelled *E. coli* by the freshwater sponge *Ephydatia fluviatilis*. Physiol. Zool. 59, 495–504.

Wilson, H.V., 1907. On some phenomena of coalescence and regeneration in sponges. J. Exp. Zool. 5, 245–257.