Increased expression of the potential proapoptotic molecule DD2 and increased synthesis of leukotriene B₄ during allograft rejection in a marine sponge

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Abstract

Sponges (*Porifera*) are a classical model to study the events during tissue transplantation. Applying the 'insertion technique' autografts from the marine sponge *Geodia cydonium* fuse within 5 days. In contrast, allografts are rejected and destroyed. Here we show that during allograft rejection the cells in the grafts undergo apoptosis; 5 days after transplantation 46% of the cells show signs of apoptosis. In a previous study it was shown that during this process a tumor necrosis factor-like molecule is induced in allo- and xenografts. Molecules grouped to the superfamily of tumor necrosis factor receptors and a series of associated adapter molecules contain the characteristic death domain. Therefore, we screened for a cDNA encoding such a domain. Here we report on the first invertebrate molecule from *Geodia cydonium* comprising a death domain. The potential proapoptotic molecule DD2, with a calculated M_r of 24 970, possesses in contrast to all known mammalian death domain-containing proteins two such domains with highest similarity to the death domain present in human Fas/APO-1. The expression of this gene is not detectable in control tissue but strongly upregulated in allografts; only very low expression is seen in autografts. Parallel with the increase of the expression of the potential proapoptotic molecule DD2 in allografts the level of LTB₄ drastically increases from 2.5 pg/mg of protein (controls) to 389 pg LTB₄/mg during a period of 5 days after transplantation; the level of LTB₄ in autografts does not change. Very likely in response to inflammatory reactions the LTB₄ metabolizing enzyme LTB₄ 12-hydroxy-dehydrogenase is expressed both in auto- and allografts. These results demonstrate that sponges are provided with apoptotic pathways, similar to those present in deuterostomes and apparently absent in protostomes, which are composed of molecules comprising a death domain. In addition, it is suggested that in sponges LTB₄ is one metabolite which is

involved in the initiation of apoptosis. It is postulated that the potential proapoptotic effect of LTB₄ is prevented in autografts by the expression of the LTB₄ 12-hydroxy-dehydrogenase. *Cell Death and Differentiation* (2000) 7, 461–469.

Keywords: sponges; invertebrates; *Geodia cydonium*; apoptosis; potential proapoptotic molecule DD2; LTB₄ 12-hydroxy-dehydrogenase

Abbreviations: aa, amino acid; LTB₄, leukotriene B₄; nt, nucleotide; PCR, polymerase chain reaction; TNF, tumor necrosis factor; TNFR, TNF-receptor

Introduction

Sponges [*phylum Porifera*] have been used since 1907¹ as a model to study the key question of immunity: how is an organism able to distinguish between self and nonself. In contrast to higher vertebrates the understanding of the molecular basis for the discrimination between self/self and self/nonself in invertebrates is still fragmentary. In vertebrates, the interplay between cytokines, eicosanoids and leukocytes controls immune reactions in a complex manner. In mammals the release of some cytokines, e.g. interleukins or tumor necrosis factors, is under the influence of eicosanoids;^{2–4} some of those cytokines cause in turn an increased synthesis of eicosanoids.^{5,6} The cytokine receptor distribution on immune cells is also mediated by eicosanoids.⁷ Hence in mammals, the eicosanoids and the cytokines affect each others function in a controlled feedback loop.

By application of molecular biological techniques it became increasingly obvious in recent years that sponges as the phylogenetic oldest, still extant metazoan phylum^{8,9} are provided with the characteristic elements for cell adhesion observed in higher metazoan phyla.¹⁰ In line with these studies it could be clarified, mainly from results with the marine sponges *Suberites domuncula* and *Geodia cydonium*, that *Porifera* have molecules similar to those known from the mammalian innate immune system, such as molecules containing scavenger receptor cysteine-rich domains,^{11,12} cytokine-like molecules¹³ or members of the (2'-5')oligoadenylate pathway.^{14–16} Furthermore, it could also be shown that 'precursors' of the second type of immune response in mammals, the adaptive immune system, are present in sponges.^{17,18}

Sponges, with their remarkable regeneration capacities,¹ react during regeneration with inflammatory responses.¹⁹ Mainly transplantation studies have been performed to understand the immune system in sponges.^{20–23} In the past we have used both, the parabiotic attachment

technique²⁴ and the insertion technique,^{24,25} to study in *G. cydonium* or *S. domuncula* the differential expression of genes involved in autograft fusion or allograft/xenograft rejection. It is known that allo(xeno)-recognition and rejection in vertebrates is closely connected with inflammation; allograft rejection involves both cytotoxic and inflammatory mechanisms.^{26,27} Recently we found that the gene encoding a molecule, which has an amino acid (aa) sequence similar to the allograft inflammatory factor 1 known to be expressed in rejecting allografts in mammals²⁸ is highly upregulated in allografts of sponges.^{17,29}

In early phases of inflammation in mammals, during graft recognition or wound healing, reactive oxygen species (ROS) are formed which are then detoxified by glutathione peroxidases (GPX).³⁰ Recently one novel non-selenium GPX, also termed antioxidant protein 2, was identified in mice, which was found to be upregulated during the early inflammatory phase of wound healing.³¹ The related enzyme was also identified and cloned in sponges and found to be upregulated in auto- and allografts.^{17,29}

In a recent study with mammalian cells it was found that overexpression of GPX results in a decreased level of leukotriene B₄ (LTB₄).³² Furthermore, LTB₄ stimulates the production of ROS in certain mammalian cells.^{33,34} LTB₄ is a potent proinflammatory factor produced in a series of mammalian tissue (reviewed in³⁵); its role in invertebrate tissue transplantation or in the process of apoptosis is not yet known. The biologically active LTB₄ is converted by two mechanisms to less active metabolites, either to 20-hydroxy/carboxy-LTB₄ by the cytochrome P-450 LTB₄ ω -oxidation³⁶ or to 12-oxo-LTB₄ in the presence of the LTB₄ 12-hydroxy-dehydrogenase, an NADP⁺-dependent enzyme.³⁷ The first mentioned inactivation process occurs in leukocytes,³⁸ while the second one has been identified in other tissues.³⁷ In consequence, it was intriguing to investigate the level of synthesis of LTB₄ in auto- and xenografts from sponge; the species *G. cydonium* was used. In the present study the LTB₄ 12-hydroxy-dehydrogenase was cloned from a *G. cydonium* cDNA library.

In human systems several signals, e.g. tumor necrosis factor (TNF), have been identified which stimulate arachidonic acid metabolism to produce prostaglandins, thromboxanes and leukotrienes, among them also LTB₄ (reviewed in³⁴). The TNF mediates its effect through interaction with receptors belonging to the TNF-receptor superfamily (TNFR) (reviewed in³⁹). Binding of TNF to the TNFR induces apoptotic cell death (see⁴⁰) through a unique cytoplasmic motif termed death domain.^{41,42} Signaling by TNFR involves adapter molecules, e.g. the TNFR-associated death domain, the Fas-associated death domain (FADD) also termed MORT1, or the receptor-interacting protein (RIP) (reviewed in⁴³). Both, the TNFRs and their adapter molecules comprise a conserved death domain (see^{40,43}). Until now neither a TNFR molecule nor one of its associated molecules have been cloned from invertebrates. Therefore, we decided to screen for a first cDNA from the invertebrate phylum Porifera which encodes for a polypeptide which comprises a death domain. This approach was successful and resulted in the isolation of a potential proapoptotic molecule DD2.

Here we report that cells in allografts of *G. cydonium* undergo apoptosis, a process which is paralleled by a strong increase of LTB₄ synthesis. In addition, it is shown that the gene of the potential proapoptotic molecule DD2 is highly expressed only in allografts while the expression of the LTB₄ 12-hydroxy-dehydrogenase is upregulated in allografts as well as in autografts from this sponge. Thus first evidence is established suggesting that LTB₄-mediated reactions and (a) TNF-like pathway(s) contribute to apoptotic reactions in sponges in a manner known from deuterostomes.

Results

Autograft fusion and allograft rejection

The 'insertion technique' was used for the experiments. If tissue from the same specimen was inserted into the host, the autotransplant fused after 5 days (Figure 1A,C). In contrast, tissue from a different specimen is rejected. This process of allograft rejection starts usually 3 days after the grafting procedure (Figure 1B,D). After this period the allografts show necrotic alterations (Figure 1D) and are completely rejected after 10 days.^{24,44,45}

Induction of apoptosis in autografts

In contrast to the autografts (Figure 1C), the allografts are rejected and show after a period of 5 days a dark brown color (Figure 1D). In an approach to clarify if the process of rejection is an active process, sections through grafts were performed after a defined period of time and analyzed for apoptosis using the TUNEL labeling assay. This technique is suitable as a first indication to demonstrate that cells undergo apoptosis.⁴⁶ As summarized in Figure 2, the percentage of apoptosis-positive cells in autografts remained below 14% during the complete observation period of 5 days. In contrast, the percentage of

Figure 1 Autograft fusion and allograft rejection in *G. cydonium*. (A) A graft was removed with a cork drill from one specimen and transplanted into a hole (H) from the host; here, the fusion reaction of an autograft (au-G) is shown. (B) Rejection of an allograft (al-G) 5 days after transplantation. (C) A section through the region of fusion of an autograft (after 5 days). (D) Cross section through the allograft; the graft shows a dark brown color. Magnifications: A and B: $\times 1.5$; C and D: $\times 0.8$

apoptosis-positive cells in allografts increased from $12 \pm 6\%$ (day zero) to $31 \pm 10\%$ already 1 day after grafting; this value increases to $46 \pm 16\%$ in allografts during the 5 days' incubation period (Figure 2). In the zones surrounding the grafts the percentage of apoptosis-positive cells remained below 15% (data not shown).

Cloning of the sponge sequence encoding the potential proapoptotic molecule GC_DD2

From earlier studies we have some evidence that a tumor necrosis factor-like protein is expressed during graft rejection.⁴⁴ Until now we did not succeed to clone the respective gene. Therefore, an alternative approach was chosen. In vertebrate cells the tumor necrosis factor binds to receptors belonging to the tumor necrosis factor receptor superfamily.³⁹ These receptors are characterized by the presence of a death domain.⁴⁰

Using a degenerate primer and applying the PCR technique, the complete cDNA from *G. cydonium*, encoding the potential proapoptotic molecule DD2, termed *GCDD2*, was isolated. It is 888 nt long and has a potential open reading frame (ORF), from the putative AUG initiation codon at nt 107–109 to the stop codon at nt 797–799, thus encoding a 230 aa long polypeptide (Figure 3). The putative start codon for Met shows a strong Kozak consensus⁴⁷ of GATCAUGG (the start AUG is underlined). The deduced aa sequence, GC_DD2, has a putative size (M_r) of 24 970 and an isoelectric point (pI) of 5.15.⁴⁸ Northern blot analysis was performed with the sponge *GCDD2* clone as a probe; one prominent band of approximately 1.1 kb was obtained, confirming that a full length cDNA was isolated (Figure 4).

The sponge potential proapoptotic polypeptide GC_DD2 displays an overall highest similarity to the human FADD (MORT1) protein^{49,50} with 11% identical and 28% similar aa

residues. Sponge DD2 comprises no potential transmembrane helix⁴⁸ (RAOARGOS). Interesting is the fact that in contrast to any other known molecule involved in the FasL/ TNF death signaling,⁴⁰ the sponge DD2 contains not only one but two death domains. In the alignment, shown in Figure 3A, the two death domain motifs from human FADD/ MORT1 and from Fas/APO-1 were aligned twice sequentially under the complete sponge sequence. The death domain from Fas/APO-1 shared with the domain I (domain II) from the sponge potential proapoptotic molecule DD2 25%²⁶ identical and 50%⁴⁷ similar aa residues; almost the same similarity is seen for FADD/MORT1, 28%¹⁵ identical and 47%⁴¹ similar aa residues. No related sequence could be detected in the (almost) complete set of *Caenorhabditis elegans* proteins (www.The Sanger Centre Web Server.ac.uk); the fact that FADD-related molecules are missing in *C. elegans* has been discussed recently.⁵¹

A phylogenetic analysis has been performed with the two sponge death domains and the single domains found in FADD/MORT1 and Fas/APO-1. The tree was rooted with

Figure 3 Sponge deduced polypeptide sequence containing two death domains, GC_DD2, which is related to human Fas-FADD proteins. (A). The *G. cydonium* sequence GC_DD2 was aligned with two consecutively arranged death domains from the human death domain-containing protein FADD/ MORT1 (DD/FADD; Q13158 and U24231; ^{49,50} the motif spans aa₁₁₁ and aa₂₁₃) and the human Fas/APO-1 (DD/APO1; Q14293; ⁶⁵ aa₂₄₄ to aa₃₁₃). The borders of the domains are marked. (B) Rooted phylogenetic tree, constructed after alignment of the two potential death domains present in the sponge sequence GC_DD2 termed GC_DD2 domain I and II) with the single death domains in FADD/MORT1 (FADD HUMAN) and Fas/APO-1 (APO1 HUMAN). The related death domain-like sequence, present in human ankyrin (ANKYRIN1 HUMAN; P16157⁷⁶ aa₁₄₀₃ to aa₁₄₈₇) was used as outgroup. The analysis was performed by neighbor-joining as described under Materials and Methods. The numbers at the nodes are an indication of the level of confidence – given in percentage – for the branches as determined by bootstrap analysis [1000 bootstrap replicates]. The scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence

Figure 2 Apoptosis in auto- and allografts. Both sections through autografts and through allografts were performed and stained for apoptosis using the TUNEL procedure. The apoptosis-positive cells are termed apoptotic cells. The percentage of apoptotic cells both in autografts (open bars) and in allografts (closed bars) are determined at time zero and after a transplantation period of 1–5 days. Approximately 200 cells were counted in five independent experiments (mean $\pm$ S.D.)

the next closest death domain-related sequence present in ankyrin.⁵⁰ The robust grouping separates the sponge death domain I and domain II from FADD/MORT1 and the ankyrin domain; both sponge domains show closer relationship to the death domain in Fas/APO-1 (Figure 3B).

Level of expression of sponge potential proapoptotic molecule DD2

In control tissue no expression of the gene encoding the potential proapoptotic molecule DD2 can be detected by Northern blotting. First low levels of transcripts (0.02 with respect to β -tubulin expression) are seen in autografts 1 day after transplantation (Figure 4A), after 3 days the expression increases to 0.05.

In contrast the expression of the potential proapoptotic molecule DD2 increases in allografts to a level of 1.3 after 1 day and even to 1.8 3 days after transplantation (Figure 4B). These data demonstrate that the steady state level of the potential proapoptotic molecule is strongly upregulated in allografts.

Level of LTB₄ in grafts

The preceding two paragraphs show that sponges contain a novel molecule, provided with two death domains. The demonstration of this sponge DD2 molecule, which is related to FADD/MORT1 and Fas/APO-1, strongly suggests that already in the lowest metazoan species a proapoptotic pathway, initiated by a tumor necrosis factor-like molecule exists.

Previous studies in mammalian systems revealed that the tumor necrosis factor causes an activation of the arachidonic acid metabolism, resulting in the production of

prostaglandins, thromboxanes and leukotrienes.⁵² During this process LTB₄, a potent chemotactic and proinflammatory factor, is produced.⁵³ Here we determined the level of LTB₄ both in autografts and in allografts. The experiments revealed that the level of LTB₄ in autografts was low, approximately 2.5 pg/mg of total protein and did not change in the grafts during a period of 5 days after transplantation (Figure 5). However, the level of LTB₄ increased drastically in allografts after transplantation. A concentration of 389 ± 42 pg LTB₄/mg is measured 1 day after transplantation, while the level decreased to $212 \pm$ pg/mg after day 5 (Figure 5).

Cloning of the LTB₄ 12-hydroxy-dehydrogenase from *G. cydonium*

The NAD(P)⁺-dependent LTB₄ 12-hydroxy-dehydrogenase shares high conservation within the α -helix segment [β -sheet- α -helix- β -sheet motif] to a series of further NAD(P)⁺-dependent dehydrogenases and also to the human LTB₄ 12-hydroxy-dehydrogenase.⁵⁴ Using a degenerate primer the sponge LTB₄ 12-hydroxy-dehydrogenase was isolated by PCR. The 1151 nt long insert of the cDNA, *GCLB4*, has an ORF of 335 aa and at nt 31–33 a putative codon for a start Met. The putative translation initiation site AUG, TCGAUGG, is of average strength.⁴⁷

The aa sequence, LB4_GEOCY, deduced from the *GCLB4* sequence encoding the putative LTB₄ 12-hydroxy-dehydrogenase is 335 aa long (Figure 6A) and corresponds to a total mass of 36 716 Da; it has a pI of 5.59. Expression of the *GCLB4* gene was examined by Northern blot analysis showing a single 1.3 kb transcript (Figure 4). The sponge LTB₄ 12-hydroxy-dehydrogenase comprises two prominent regions: First, the β -sheet- α -helix- β -sheet structure which is crucial for the activity of the hydroxy-dehydrogenase and conserved also in other NAD(P)⁺-dependent enzymes.⁵⁴ Second, a 'homeobox' domain signature is found between aa₂₆₈ and aa₂₉₁ in the sponge

Figure 4 Level of transcripts of the potential proapoptotic molecule DD2 and LTB₄ 12-hydroxy-dehydrogenase in grafts from *G. cydonium*. RNA from autografts (A) or allografts (B), 0 (Con) to 3 days (lanes a to c) after transplantation, has been extracted, size-separated, blot transferred and probed with labeled probes for the potential proapoptotic molecule (*GCDD2*) or the LTB₄ hydroxy-dehydrogenase (*GCLB4*). The intensities of the bands corresponding to the transcripts of the potential proapoptotic molecule, abbreviated DD2, and the hydroxy-dehydrogenase, LH4 OH-DH, were correlated with those seen with β -tubulin; further details are given in the text

Figure 5 Changes of tissue LTB₄ levels during graft recognition. The LTB₄ metabolites have been extracted from both autografts (open bars; below 4 pg/mg) and allografts (closed bars) after the indicated period of time after transplantation. Values are expressed as means ($\pm$ S.D.); n =five independent experiments

Figure 6 Sponge LTB₄ 12-hydroxy-dehydrogenase. (A) Alignment of the deduced *G. cydonium* sequence, LB4_GEOCY, with the human LTB₄ 12-hydroxy-dehydrogenase (LB4D_HUMAN, accession number Q14914;⁵⁴). Similar aa (with the same physico-chemical properties) between the two sequences are shown in inverted type. The characteristic segments of the LTB₄ 12-hydroxy-dehydrogenases are indicated, the β -sheet– α -helix– β -sheet motif, the ‘homeobox’ domain signature (the aa residues forming the consensus are marked [arrow head]) and the Pro-rich motif. (B) Rooted phylogenetic tree, constructed from the sponge (LB4_GEOCY), the LTB₄ 12-hydroxy-dehydrogenase (LB4D_HUMAN) and the corresponding metazoan sequences from *Sus scrofa* (LB4D_PIG, D49386;⁵⁴ and the dithiolethione-inducible gene-1 from *Rattus norvegicus* (DIG1_RAT, P97584;⁷⁷). The YfmJ deduced aa from *Bacillus subtilis* (YFMJ_BACSU, D86417;⁷⁸) was used as outgroup. The analysis was performed by neighbor-joining as described under Materials and Methods. The numbers at the nodes are an indication of the level of confidence (given in percentage) for the branches as determined by bootstrap analysis (1000 bootstrap replicates). The scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence

sequence (Figure 6A); however, at this position the mammalian dehydrogenases display a helix–turn–helix type structure. No homology to hitherto known families and classes of homeodomains exists in the databases. Interesting is the fact that the Pro-rich motif, which is present in the COOH-terminus of the mammalian enzymes (in the dehydrogenase within aa₂₅₀ and aa₂₅₇; Figure 6A) is missing in the sponge molecule. The Pro-rich motif is assumed to be involved in the signal transduction pathway originating from a receptor tyrosine kinase by binding to *src* homology domains.⁵⁵

A phylogenetic analysis has been performed. On aa level the sponge sequence was found to be identical (similar) to the vertebrate sequences with a degree of $\approx 48\%$ ($\approx 65\%$). Construction of the rooted tree revealed that the sponge LTB₄ 12-hydroxy-dehydrogenase is the common ancestor to the available mammalian dehydrogenases from rat, human and pig (Figure 6B).

Expression of sponge LTB₄ 12-hydroxy-dehydrogenase during auto- and allograft recognition

The expression of the sponge LTB₄ 12-hydroxy-dehydrogenase increases both in autografts and in allografts almost to the same extent (Figure 4). One (three) day(s) after transplantation the level of expression is upregulated from 0.05/0.06 (control) to 0.2 (0.1) in autografts (Figure 4A); in allografts the degree of expression further increases and reaches a value of 0.3 after day 3 (Figure 4B).

It is interesting to note that in contrast to the observed increase in the expression of the LTB₄ 12-hydroxy-dehydrogenase gene both in autografts and in allografts the level of LTB₄ rises only in allografts (Figure 5). Hence, the increased expression of LTB₄ 12-hydroxy-dehydrogenase correlates only in allografts with the increase of the amount of LTB₄.

Discussion

Previous studies suggested that rejection of allo- and xenografts in the sponge system is mediated by a TNF-like molecule.⁴⁴ However, until this study no gene could be isolated from an invertebrate species which supported these earlier results, obtained by application of immunological techniques. In the present study the ‘insertion technique’ was applied to demonstrate that allografts from *G. cydonium* are rejected, while autografts from this species fuse. Applying this technique it is shown that during rejection the allografts undergo apoptosis.

During the active process of apoptosis, a molecule is expressed in allografts which comprises two death domains. In autografts this gene is only marginally expressed. Since molecules comprising death domains are, until now, only found in proteins involved in the TNFR-mediated apoptotic pathways (see Introduction), the sequence deduced from the cDNA from *G. cydonium* was termed potential proapoptotic molecule DD2. This novel molecule shares highest sequence similarity to the death domain of Fas/APO-1. In humans death receptor Fas/APO-1 is involved in three types of apoptosis; deletion of activated mature T cells, killing of targets, e.g. virus-infected cells, and killing of inflammatory cells.⁵⁶ Signaling by Fas/APO-1 is – like in the TNFR pathway – transduced by FADD, resulting in an activation of caspase 8 and ultimately in the induction of apoptosis.⁴³ It is interesting to note here that *C. elegans* does not contain a molecule with a considerable similarity to the death domain, again supporting the view that sponges comprise a large set of molecules, especially of immune molecules^{17,18} in common with deuterostomes but not present in the protostomian *C. elegans*. Until now we have not been successful to find further death domain-containing molecules, similar to those of higher deuterostomes, e.g. TNFR.

Parallel with the upregulation of the sponge potential proapoptotic molecule DD2, and increase of LTB₄ synthesis takes place. In mammalian systems, LTB₄ augments immune and inflammatory responses⁵⁷ and controls regulation of cell proliferation.⁵⁸ Cells in allografts produce

large amounts of LTB₄, suggesting that this molecule is involved in the induction of apoptosis. It has been reported that LTB₄ causes a strong effect on intracellular Ca²⁺ mobilization,³⁷ followed by the production of reactive oxygen species.⁵⁹ The reactive oxygen species and superoxide anions are thought to be involved in killing of pathogenic organisms and in a localized damage of affected tissues.³⁴ Since it is established that reactive oxygen species can also cause apoptosis in mammalian systems,⁶⁰ we suggest that the latter effect is the major one in sponges.

Interesting is the finding that the level of LTB₄ in autografts of *G. cydonium* remained unchanged after grafting in spite of the fact that also in these grafts inflammatory responses proceed.¹⁷ This fact can be explained by the finding that immediately after autotransplantation the 'detoxifying' enzyme, the LTB₄ 12-hydroxy-dehydrogenase, is induced. This enzyme is known to convert LTB₄ to 12-oxo-LTB₄; 12-oxo-LTB₄ is a biologically less active compound.³⁷ In addition, 12-oxo-LTB₄ acts as a LTB₄ receptor antagonist and blocks the binding of LTB₄ to the corresponding receptor.⁵⁴ Therefore, it is plausible that the effects of the possible intermediately synthesized LTB₄ in autografts is rapidly abolished on two levels, by conversion to 12-oxo-LTB₄ and in turn by blocking the LTB₄ receptor. Preliminary experiments with 12-oxo-LTB₄ did not show any effect on Ca²⁺ mobilization (at a concentration of 1 μM) using cells from *G. cydonium* as described for human leukocytes³⁷ (data not shown).

In summary, the identification of the potential proapoptotic molecule DD2 in the sponge *G. cydonium* suggests that apoptotic pathways, related to those of Fas/APO-1 and TNFR, exist already in sponges. In addition, the data suggest that LTB₄ is involved in the initiation/maintenance of the apoptotic process in allografts of these animals. The postulated proapoptotic effect of LTB₄ is prevented by the expression of the LTB₄ 12-hydroxy-dehydrogenase which might convert LTB₄ to 12-oxo-LTB₄. Work is currently in progress in our laboratory to investigate the biological activity of LTB₄ and 12-oxo-LTB₄ on Ca²⁺ metabolism and apoptosis.

Materials and Methods

Materials

Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained as described earlier.^{25,61} DIG (digoxigenin) DNA labeling kit, DIG-11-dUTP, anti-DIG AP Fab fragments, CDP-Star (disodium 2-chloro-5-(4-methoxyispiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo(3.3.1.1^{3,7})decan}-4-yl)phenyl phosphate) were obtained from Boehringer Mannheim (Mannheim; Germany). The *in situ* cell death detection kit/Fluorescein from Boehringer Mannheim (Boehringer Manneheim). The LTB₄ enzyme-immunoassay system was obtained from Amersham (Little Chalfont, Buckinghamshire HP7 9NA; UK).

Sponges

Specimens of the marine sponge *Geodia cydonium* (Porifera, Demospongiae, Geodiidae) were collected in the Northern Adriatic

near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) at a temperature of 17°C.

Allo- and autografting procedure

For the grafting experiments described here, the 'insertion technique' was used.^{24,45} Tissue pieces from the same (autograft) or from a different specimen (allograft) were removed with a cork drill (diameter of 1 cm; approximate length of 4 cm) and inserted into holes of the host, which had a slightly narrower diameter (0.9 cm). The sponges were kept for up to 5 days to be subsequently analyzed.

After 5 days the grafts were analyzed by cutting them at the transplantation area and inspected with an Olympus OSP-MBI binocular microscope.

Cell death assay

DNA fragmentation (TUNEL staining) was determined using the *in situ* cell death detection kit (Boehringer Mannheim). Fresh tissue was fixed in 4% paraformaldehyde; after dehydration the samples were embedded in Technovit 8100⁶² as described before,²⁴ according to the instructions of the manufacturer. Sections of 2 μm thickness were prepared. The TUNEL-stained cells were counterstained with propidium iodide (5 μg/ml) and visualized by fluorescence microscopy; the percentage of apoptosis [TUNEL]-positive cells are given.

Determination of LTB₄

Graft tissue was removed after the indicated periods of auto- and allotransplantation. Approximately 0.5 g of tissue was rinsed in saline, supplemented with 10 mM of ethylenediaminetetraacetic acid, and frozen in liquid nitrogen. The frozen tissue was homogenized with cold ethanol; after centrifugation the precipitate was used for the determination of protein content while the supernatant was used for the measurement of LTB₄.⁵⁷ The supernatant was acidified and passed through a octadecyl C-18 silica gel column (Baker; Phillipsburg, NJ, USA); the LTB metabolites were eluted with ethyl acetate.⁵⁷ The eluate was dried down and the residue was dissolved in the RIA buffer and assayed for the concentrations of LTB₄ using the 'LTB₄ enzymeimmunoassay system'. The values (working range 20–900 pg/ml) have been corrected by application of an internal standard, [³H]-LTB₄, which had been added at the beginning of the isolation procedure; LTB₄ concentrations have been read from a standard curve. The levels of LTB₄ are correlated to the amount of protein, present in the respective starting material. The protein content was determined using the Lowry method.⁶³

PCR cloning of the putative sponge proapoptotic molecule DD2 and LTB₄ 12-hydroxy-dehydrogenase

The complete sponge cDNA, encoding the putative proapoptotic molecule DD2, termed *GCDD2*, was isolated from the *G. cydonium* cDNA library⁶⁴ by polymerase chain reaction (PCR). The degenerate forward primer was directed against the conserved aa segment within the conserved region of the human death domain Fas/APO-1 (aa₂₅₇ to aa₂₆₅; Q14293;⁶⁵ the primer 5'-GCIAAIATIGAT/CGAA/GATT/CAAA/GAAIGAT/C-3' (where I=inosine), in conjunction with the ZAPII 3'-end vector-specific primer T7 was used. The PCR reaction was carried out using a GeneAmp 9600 thermal cycler (Perkin Elmer) as described⁶⁶ applying an initial denaturation temperature of 95°C for 3 min, then 32 amplification cycles at 95°C, for 30 s, 51°C for 45 s, 74°C for 1.5 min, followed by a final extension step at 74°C for 10 min. A fragment of

≈700 bp was used to isolate the cDNA from the library by primer walking.⁶⁷ The longest insert obtained had a size of 1888 nt (excluding the poly(A) tail). The clone was termed *GCDD2* and sequenced using an automatic DNA sequenator (Li-Cor 4200).

The complete sponge cDNA, encoding the putative LTB₄ 12-hydroxy-dehydrogenase, termed *GCLB4*, was isolated from the *G. cydonium* cDNA library by the same strategy. Here, the forward primer was directed against the conserved aa segment within the α-helix segment [β-sheet–α-helix–β-sheet motif] of the human LTB₄ 12-hydroxy-dehydrogenase (554), aa₁₅₈ to aa₁₆₆, 5'-GTIGGICAA/GATT/C/AGCIAAA/GCTIAAA/GGG-3' (where I=inosine); an annealing temperature of 56°C was used. The insert has a size of 1151 nt.

Sequence comparisons

The sequences were analyzed using computer programs BLAST⁶⁸ and FASTA.⁶⁹ Multiple alignments were performed with CLUSTAL W Ver. 1.6.⁷⁰ Phylogenetic trees were constructed on the basis of aa sequence alignments by neighbor-joining, as implemented in the 'Neighbor' program from the PHYLIP package.⁷¹ The distance matrices were calculated using the Dayhoff PAM matrix model as described.⁷² The degree of support for internal branches was further assessed by bootstrapping.⁷¹ The graphic presentations were prepared with GeneDoc.⁷³

Northern blotting

RNA was extracted from liquid-nitrogen pulverized sponge tissue with TRIzol Reagent (Gibco BRL) as recommended by the manufacturer. For extraction autografts or allografts have been used. An amount of 5 µg of total RNA was electrophoresed through formaldehyde/agarose gel and blotted onto Hybond N⁺ membrane following the manufacturer's instructions (Amersham). Hybridization experiments were performed with DNA probes from *G. cydonium* potential proapoptotic molecule DD2, *GCDD2* (a segment of ≈600 bp was used), *G. cydonium* LTB₄ 12-hydroxy-dehydrogenase *GCLB4* (≈600 bp), and *G. cydonium* β-tubulin⁷⁴ *GCBTUB* (≈600 bp). The probes were labeled with DIG-11-dUTP by the DIG DNA labeling kit. Hybridization was performed with the antisense DIG-labeled probes over night following the instructions of the manufacturer (Boehringer). After washing DIG-labeled nucleic acid was detected with anti-DIG Fab fragments and visualized by the chemiluminescence technique using CDP-Star, according to the instructions of the manufacturer.⁴⁵

To quantitate the signals of the Northern blots the chemiluminescence procedure was applied.⁷⁵ The screen was scanned with the GS-525 Molecular Imager (Bio-Rad). The relative values for the expression of sponge potential proapoptotic molecule DD2 and LTB₄ 12-hydroxy-dehydrogenase in *G. cydonium* are correlated with the intensities of the bands measured for the expression of the tubulin gene.

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