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FACIT collagen (1α-chain) is expressed by hemocytes and epidermis during the inflammatory response of the ascidian *Ciona intestinalis*

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Summary
Based on previous cloning and sequencing study, real-time PCR and *in situ* hybridization assays of the inflamed body wall of LPS-injected *Ciona intestinalis* showed the enhanced gene expression of a collagen with FACIT structural features (*Ci*-type IX-Col 1α-chain). By using specific antibodies raised against an opportunely chosen *Ci*-type IX-Col synthetic peptide, the fibroblast property of hemocytes challenged *in vitro* with LPS (at 4 h) was displayed by flow cytometry, while immunocytochemistry identified hemocytes with large granules (morula cells) as collagen-producing cells. Hemocyte lysate supernatant analyzed in immunoblotting contained a 60 kDa band identifiable as 1α-chain-*Ci*-type IX-Col. Observations of body wall sections (immunohistochemistry method) supported the role of hemocytes and showed that epidermis expressed *Ci*-type IX-Col 1α-chain in the time course of the inflammatory reaction (within 24 h). Transcript and protein were mainly found in the epidermis that outlined the proximal side of the tunic matrix (at 24 h after LPS injection), in cells associated with the epidermis at 4 and 192 h. In conclusion, the *C. intestinalis* inflammatory response to LPS challenge appeared to be composed of a complex reaction set, and for the first time we showed in ascidians a granulation tissue with FACIT-collagen production that could participate in inflammation and wound healing. Like in vertebrates, *C. intestinalis* acute inflammatory reactions result in a regulated pattern of tissue repair with collagen expression during remodelling. *Ci*-type IX-Col could be involved in a network of non-fibril-forming collagens that participates in the organization of extracellular matrix and defense responses.

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1. Introduction

Collagens are major structural components of the extracellular matrix in tissues of vertebrate and invertebrate organisms where they are also involved in defense responses, including reparative processes [1–3]. In vertebrate acute inflammatory reactions, cellular, humoral, and molecular events are activated, resulting in a regulated pattern of tissue repair with collagen fiber bundles increasingly organized during remodelling [4–6]. In invertebrates, several collagen types, including fibril-forming and non-fibrillar collagens, have been found [7–14]. In tunicates, which occupy a key position in chordate phylogenesis [15,16], the presence of collagens has been shown by histochemical and biochemical methods [17]. Recently, in *Ciona intestinalis*, a cDNA cloned from pharynx tissue revealed a type IX collagen (*Ci*-type IX-Col 1α-chain) characterized by three short triple-helical domains interspersed with four non-triple-helical sequences and structural features of fibril-associated collagens with interrupted triple helices (FACIT) [18]. The 1α-chain sequence is characterized by 186 Gly–X–Y repeats in three domains. The proposed domain diagram shows a triple-helical structure (COL1, COL2, and COL3) and a non-collagenous domain (NC1, NC2, NC3, and NC4) [18]. In the UCSC Genome Bioinformatics Site, a Ci-type IX-Col 1α-chain nucleotide sequence can be found in the *C. intestinalis* genome (identified position chr01p:4171494–4180671). Comparative analyses showed amino-acid sequence homology with mouse (47.64%) and human (48.95%) type IX collagen 1α-chain [18], suggesting that, similar to mammals, it may be involved in forming a network of non-fibril-forming collagens that participates in the organization of extracellular matrix and defense responses.

A strong inflammatory reaction was found in the tunic of this ascidian as a response to the injection of erythrocytes [19,20], foreign proteins [21], and LPS [22]. Numerous hemocytes of various types promptly infiltrated the tunic tissue, where they released their granule or vacuole contents. Within 2–3 days, a capsule that included the inflamed tissue became visible through the transparent tunic at the injection site. Cell-mediated inflammatory events included a tunic repair response. In several experiments, a variable percentage of treated ascidians presented tissue damage and, 5–8 days after the challenge, a wound repair phase appeared as a thick capsule. In a previous paper, we showed that collagen fibers could be identified by monoclonal antibodies raised against bovine Type I collagen [23].

The present study was mainly focused on tissues and cells which express *Ci*-type IX collagen (1α-chain) in *C. intestinalis* inflamed body wall by using real-time PCR, *in situ* hybridization assay, immunocytochemical and immunohistochemical methods, as well as hemocyte flow cytometry analysis. Results are consistent in showing gene expression and enhanced *Ci*-type IX collagen after intratunic LPS injection. Prompt expression of *Ci*-type IX-Col 1α-chain gene and enhanced collagen in the epidermis and pharynx hemolymph vessels characterized the response of the pharynx wall to LPS. In addition, flow cytometry analysis showed that hemocytes from the hemolymph promptly became activated and expressed FACIT collagen 1α-chain when stimulated *in vitro*. We demonstrated for the first time that the ascidian inflammatory response includes a granulation phase with FACIT-collagen expression presumably involved in remodelling the extracellular matrix, the fibroblast-like role of hemocytes (morula cells), and the inflammatory response of the epidermis.

2. Materials and methods

2.1. Tunicates

Ascidians were collected from Sciacca Harbour (Sicily, Italy), maintained in tanks with aerated sea water at 15 °C, and fed every second day with a marine invertebrate diet coraliquid (Sera, Heinsberg, Germany).

2.2. Hemolymph collection and hemocyte suspension preparation

To collect hemocytes, hemolymph was harvested into a two-fold excess of ice-cold sterile anticoagulant solution (11 mM KCl, 43 mM Tris-HCl, 0.4 M NaCl, 10 mM EDTA, pH 7.4). The hemolymph was withdrawn from the heart with a sterile syringe containing anticoagulant solution (1:2) and immediately centrifuged at 800*g* (10 min, 4 °C). After centrifugation at 800*g* for 10 min at 4 °C, hemocytes were washed with ice-cold sterile anticoagulant solution and re-suspended in marine solution (MS: 12 mM $CaCl_2 \cdot 6H_2O$, 11 mM KCl, 26 mM $MgCl_2 \cdot 6H_2O$, 43 mM Tris-HCl, 0.4 M NaCl, pH 8.0) isosmotic with hemocytes (1090 mOsm/kg). Cells were counted using an improved Neubauer chamber. Dead hemocytes were estimated by Trypan blue (0.05% final concentration in MS) exclusion test and values lower than 2.0% were found.

All the solutions were filtered through a 0.22 μm filter (Millipore, USA) and autoclaved.

2.3. LPS injection

Lipopolysaccharide (*Escherichia coli* 055:B5, LPS) solution was prepared in sterile MS. 100 μl of an LPS solution (100 μg LPS per animal) was injected into the tunic tissue at the median body region. Ascidians, both untreated and injected with MS (100 μl), were used as controls.

2.4. Body wall explant preparation and histology

The tunic surface was cleaned and sterilized with ethyl alcohol, and body wall fragments (200 mg) containing both tunic and pharynx tissue were excised from the injection site at various times after the injection (1–192 h).

For immunohistochemistry and *in situ* hybridization studies, body wall fragments were fixed in Bouin's fluid (saturated picric acid:formaldehyde:acetic acid 15:5:1) for 24 h, paraffin embedded, and serially cut at 6 μm (Leica RM2035 microtome, Solms, Germany).

2.5. Real-time PCR analysis

Tissue expression of the *Ci*-type IX-Col gene was detected by real-time PCR using the Taqman method. Primers and hybridization probes were designed using Primer Express software V.0 and synthesized commercially (Applied Biosystems, Foster City, USA). The Taqman probe sequence contained a 5′_FAM fluorophore and 3′_MGB quencher for the target gene (*Ci*-type IX-Col 1α-chain Accession Number AY619995) and 5′_VIC fluorophore and 3′_MGB quencher for the housekeeping gene (*C. intestinalis* actin Accession Number AJ297725). Real-time PCR analysis was performed using the Applied Biosystems 7500 real-time PCR system. Tissue expression was performed in a 25 μl PCR reaction containing 2 μl cDNA converted from 250 ng of total RNA, 100 μM *Ci*-type IX-Col probe, 400 μM *Ci*-type IX-Col forward (5′-GGCGCTTCTGCTTGTAACG-3′) and reverse primers (5′-TGCTCTTCCGGAGCTTCTTTC-3′), 100 μM actin probe, 400 μM actin forward (5′-TGATGTTGCCGCACTCGTA-3′) and reverse (5′-TCGACAATGGATCCGGT-3′) primers, and 12.5 μl of Taqman PCR Master Mix (Applied Biosystem). The 50 cycles of the two-step PCR program consisted of initial polymerase activation for 3 min at 95 °C followed by denaturing at 95 °C for 15 s, and annealing/extension at 60 °C for 45 s, in which the fluorescent signal was detected. Each set of samples was run three times and each plate contained quadruplicate cDNA samples and negative controls.

To obtain sample quantification, the $2^{-\Delta\Delta Ct}$ method was used and the relative changes in gene expression were analyzed as described in the Applied Biosystems Use Bulletin N.2 (P/N 4303859). For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and reference gene must be approximately equal. To assess whether two amplicons have the same efficiency, a sensitive method was used for checking how ΔCt varied with template dilution. In this respect, serial dilutions (1.0, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01) of cDNAs were amplified by real-time PCR using target and housekeeping gene-specific primers, and the ΔCt, i.e. $CT_{(Ci\text{-type IX-Col})} - CT_{(Actin)}$, was calculated, in three replicates, for each cDNA dilution. Data were fit using least-squares linear regression analysis. The amount of type IX collagen transcript from different tissues was normalized to actin in order to compensate for variations in input RNA amounts. Relative *Ci*-type IX-Col expression was determined by dividing the normalized value of *Ci*-type IX-Col in each tissue by the normalized value obtained from the untreated tissue.

2.6. Anti-*Ci*-type IX-COL2 antiserum production

A peptide (16 aa, CGESGPRGVPGLQGPP) from the COL2 deduced amino-acid sequence of previously cloned *Ci*-type IX-Col 1α-chain [18] was selected by antigen-prediction programs and synthesized by Sigma-Genosys (UK). The *Ci*-type IX collagen 1α-chain showed significant homology with human and mouse 1α chain type IX collagen (47% and 48%, respectively), whereas lower homologies with type I collagen from *Paracentrotus lividus*, mouse, human, and other mammal collagen types (I, VII, VIII, X, XII, XIV, XVI, XVIII, and XIX) were found [18]. To determine if the amino-acid sequence of the COL2 peptide used for producing antibodies had similarity to annotated *C. intestinalis* proteins, a search with BLAST in *C. intestinalis* genome sequences (JGI V2), as well as in the EMBL gene bank, was carried out. Anti-COL2-peptide specific antiserum was raised in rabbits (Sigma-Genosys, UK).

2.7. Indirect peptide ELISA and competitive ELISA were used to determine the anti-*Ci*-type IX-COL2 antiserum specificity

The peptide ELISA was conducted as described by Plagemann [24]. In brief, the wells of Nunc Maxisorp ELISA plates (Nunc, Denmark) were coated overnight with the peptide dissolved in carbonate buffer, pH 9.6, at 10 μg/well. After peptide coating, the wells were rinsed with PBS containing 0.1% (v/v) Tween 20 (PBS-T) and then incubated with blocking buffer composed of PBS-T and 1% (w/v) bovine serum albumin (BSA) at room temperature for 1 h. They were then incubated with anti-*Ci*-type IX-COL2 antiserum diluted (1:1000–1:6000) in blocking solution or pre-immune rabbit serum in blocking solution (generally 1:50–1:200) at room temperature for 1 h. After rinsing with PBS-T, the wells were incubated with peroxidase-conjugated anti-rabbit IgG diluted 1:1000 in blocking solution at room temperature for 60 min, rinsed four times with PBS-T, and then incubated with *o*-phenylenediamine (2 mg in sodium citrate 0.1 M, pH 4.0) substrate (100 μl/well) for 15–30 min. The peroxidase product was quantified by measuring absorbance at 492 nm with an automatic plate reader.

To check for specificity of the antiserum, the antibody binding to immobilized peptide was inhibited by COL-2 peptide in solution (competition ELISA). The peptide-coated plates were prepared as reported above: after rinsing with PBS-T and treatment with blocking solution, the plates were incubated with a mixture containing 100 μl antiserum diluted 1:3000 or 1:5000 in PBS-T with the peptide (10 μg/well). The wells were rinsed thrice with PBS-T and the standard procedure was used as previously described.

2.8. Total RNA extraction and cDNA synthesis

To examine *Ci*-type IX-Col mRNA expression, tissue fragments (200 mg) were excised at various times (from 1 to 192 h), immediately soaked in RNAlater tissue collection solution (Ambion, Austin, TX), and stored at −80 °C.

Total RNA was isolated from ascidian tissues by using an RNAqueous™-Midi Kit purification system (Ambion) and reverse-transcribed by the Kit Ready to Go T-primed first strand using random primers (Amersham-Pharmacia Biotech, USA).

2.9. Hemocyte *in vitro* challenge with LPS and flow cytometry analysis

Hemocytes were washed twice at 800*g* (10 min, 4 °C) and cultured (1×10^6 cells/well, for 1, 2, 4, and 8 h at 18 °C) in the wells of 24-well culture plates (Corning, USA) in MS enriched with 5% RPMI 1640 medium, or supplemented with 1 μg/ml LPS. To block intracellular protein exocytosis and enhance the detection of intracellular proteins before

stimulation, the cells were pre-incubated for 1 h with 30 μM monensin (Bioscience, USA). At the specified culture times, the cells were harvested and distributed into Falcon 2054 polystyrene round-bottom tubes (Becton & Dickinson Co., Mountain View, CA). The cells were washed twice by centrifuging (800*g*) for 10 min, re-suspended in 100 μl PBS, and fixed with 4% paraformaldehyde for 20 min at room temperature. Cell permeabilization was performed by washing twice with PBS containing fetal calf serum (1%) and saponin (0.1%). The cells were incubated with anti-*Ci*-type IX-COL2 primary antibodies diluted 1:100 in PBS containing fetal calf serum and saponin, washed twice with PBS containing fetal calf serum and saponin, and incubated in the dark with anti-rabbit IgG-phycoerythrin diluted 1:100, or with isotype controls for 20 min at room temperature. Then the cells were examined with a FACScan flow cytometer (Becton Dickinson) equipped with CellQuest software. In all, 50,000 events were acquired in each analysis.

2.10. Hemocyte lysate preparation (HLS)

Hemocytes were re-suspended in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with a protease inhibitor cocktail (pepstatin A, E-64, bestatin, leupeptin, aprotinin, and AEBSF) and incubated on ice for 20 min. After incubation, they were shaken vigorously three times. Hemocyte lysates were centrifuged at 14,000*g* for 20 min at 4 °C and supernatants held at −80 °C.

Protein content was measured by the Bradford method [25] using BSA as a standard.

2.11. Western blot analysis

Following SDS-PAGE (12% acrylamide), according to the Laemmli method [26], of the hemocyte lysate supernatant (2 μg/well protein content), the gel was soaked in transfer buffer (20 mM Tris, 150 mM glycine, pH 8.8) for 10 min and proteins were transferred (1 h at 210 mA) to a nitrocellulose sheet in transfer buffer. The filter was soaked for 2 h in blocking buffer (0.14 M NaCl, 0.2 M phosphate-buffered saline, pH 7.4) containing 2% BSA and 0.05% Tween-20, incubated with anti-*Ci*-type IX-COL2 antibodies (1:1000 in blocking buffer) for 1 h, washed with blocking buffer, and incubated for 1 h with anti-rabbit IgG-alkaline phosphatase conjugate 1:8000 in blocking buffer). Finally, the nitrocellulose sheet was washed with PBS and assayed with a mixture of 3 ml of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system.

2.12. Immunocytochemistry and immunohistochemistry methods

A hemocyte preparation (5×10^5 in 100 μl) was placed on a Super Frost microscope slide for 30 min at 18 °C and fixed with 100 μl isotonic solution (0.01 M Tris-HCl, 0.5 M NaCl, 4% paraformaldehyde, pH 7.4, ISO) for 30 min at 18 °C. Cells were washed with PBS-T and treated at room temperature in a moist chamber (3 h) with 5% BSA in PBS-T and incubated overnight at 4 °C with anti-*Ci*-type IX-COL2 polyclonal rabbit antiserum diluted 1:100 in blocking buffer. After washing, the cell preparation was incubated (2 h at room temperature) with alkaline-phosphatase-conjugated anti-rabbit IgGs diluted 1:10,000 in blocking buffer, washed three times for 10 min at room temperature with PBS-T, and incubated for 1 h with 1 mM levamisole in PBS-T. Finally, the cell preparation was incubated in BCIP/NBT liquid substrate system and observed under a Leica DMLB microscope (Solms, Germany). Hemocyte types were identified according to Parrinello et al. [27].

Histological sections (6 μm) were incubated overnight at 4 °C with diluted (1:1000 in PBS-T with 1% BSA) anti-COL2 antiserum. After exhaustive washing with PBS-T, secondary anti-rabbit-peroxidase conjugate antibodies diluted 1:500 in blocking buffer were added, and the sections were incubated for 1 h at room temperature. After washing in PBS-T, the sections were treated with diaminobenzidine (DAB) buffer (50 mM Tris, pH 6.8, 30% H_2O_2), and examined under a Leica DMLB microscope (Solms, Germany). Controls were carried out by substituting primary antibodies with PBS-T or with pre-immune rabbit IgGs (Sigma, Genosis, UK).

To show that antibodies can bind collagen in the tissues, histological sections were pre-treated with 15 U of *Clostridium histolyticum* type I collagenase in 10 mM KCl, 15 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5, at 37 °C for 3 h and at room temperature overnight. After rinsing, the standard procedure was used as described previously.

Body wall tissues and cells were identified according to Millar [28] and De Leo et al. [29].

2.13. In situ hybridization assay (ISH)

ISH was carried out according to Le Guellec [30], with digoxigenin-11-UTP-labelled riboprobes (1 μg/ml final concentration) according to the manufacturer's instructions (Roche Diagnostics). The re-hydrated histological sections were digested with proteinase K (10 μg/ml) in PBS, washed with PBS-T, and treated for hybridization with 50% formamide, 5× SSC (1× SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7), 50 μg/ml heparin, 500 μg/ml yeast tRNA, and 0.1% Tween 20, at 37 °C overnight. After exhaustive washing in PBS-T and 4× SSC (twice for 10 min), the sections were incubated for 1 h with anti-DIG FAB-AP diluted 1:500 and washed in PBS-T. Finally, the sections were incubated in the 5-BCIP/NBT liquid substrate system. Color development was stopped after 30 min at room temperature.

2.14. Statistical methods

Student's *t*-test was used to estimate statistical significance. Multiple comparisons were performed with one-way analysis of variance (ANOVA) and the comparison between different groups was done using the Tukey *t*-test. Standard deviations were calculated based upon at least three experiments, each of them analyzed in triplicate. $P < 0.01$ was considered statistically significant.

2.15. Chemicals and products

Unless otherwise indicated, all chemicals, products and antibodies were from Sigma-Aldrich, Germany.

3. Results

3.1. Relative expression of *Ci*-type IX-Col 1α-chain mRNA detected by real-time PCR analysis in body wall explants

In previous analyses, the relative efficiency plots of the target gene amplification (*Ci*-type IX collagen) and internal control (actin) were examined with real-time PCR and Taqman detection. Serial dilutions of cDNA were amplified using gene-specific primers. The plot of the log cDNA dilution versus ΔCt (Figure 1) confirmed they had similar amplification efficiency, the absolute value of the slope being close to zero (0.045).

Quantitative *Ci*-type IX 1α-chain collagen mRNA expression in the body wall fragments from 32 LPS-injected ascidians showed enhanced levels in mRNA expression (Figure 2). To analyze the kinetics of gene expression from 1 to 192 h, body wall fragments (200 mg) from 4 ascidians at each time point were excised from the inflamed tissue. The enhancement was significant when compared to the results obtained by analyzing explants from the same number of untreated and MS-injected ascidians, which maintained low expression levels until 192 h (Figure 2). The transcript was detected 1 h after LPS stimulation ($P<0.01$), reached the highest significant value at 4 h ($P<0.001$), and decreased at 8 h. The gene expression had a low increase that reached its maximum at 24 h ($P<0.01$) and decreased to the control levels by 48 h.

3.2. Specificity of the anti-*Ci*-type IX-COL2 1α-chain antiserum

Polyclonal antibodies against a COL2 peptide were prepared. The homology search with BLAST in *Ciona* genome sequences and EMBL gene bank of the COL2 peptide sequence chosen by using an antigen-prediction program did not show any similarity with other known *C. intestinalis* protein sequences, whereas it revealed 93.3% similarity with

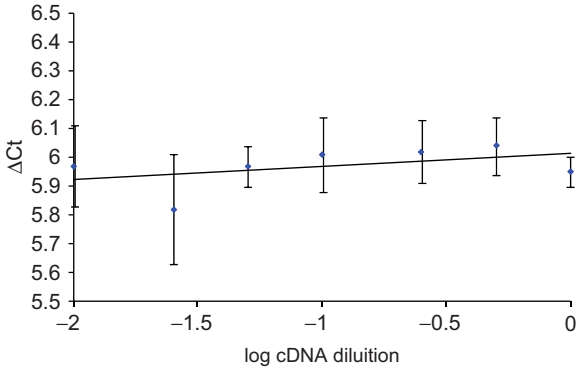

Figure 1 Relative efficiency plot of amplification of target gene (*Ci*-type IX collagen 1α-chain) and internal control (actin) examined by real-time PCR and Taqman detection. Serial dilutions of cDNA were amplified using gene-specific primers. The ΔCt ($CT_{(Ci\text{-type IX-collagen})}$-$CT_{(actin)}$) was calculated for each cDNA dilution. The data were fit using least-squares linear regression analysis ($y = 0.045x+6.0123$, $R^2 = 0.1966$). $N = 3$.

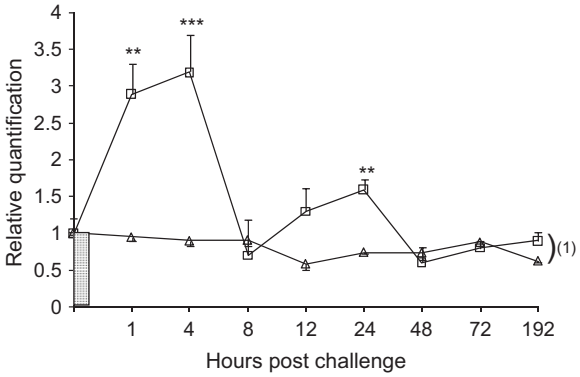

Figure 2 Real-time PCR analysis. Time course of *Ci*-type IX-collagen 1α-chain gene expression in *Ciona intestinalis* pharynx explants following 100 μg LPS injection/ascidian (—□—) into the tunic tissue at the median body region, compared to gene expression in ascidians injected with 100 μl marine solution (—Δ—). Values, plotted as mean ± SD, were inferred from four ascidians examined in distinct experiments, and each assay was performed in triplicate. Significance was evaluated by comparing the values with the expression level of explants from four untreated ascidians (▨). $^{**}p<0.01$, $^{***}p<0.001$. (1) Multiple comparisons were performed with one-way analysis of variance (ANOVA) and the comparison between different groups was done by the Tukey *t*-test.

chicken collagen alpha-1(IX) chain precursor (P12106). Although the peptide sequence can only be an indicator of the epitope structure, the lack of similarity between the peptide sequence and known *C. intestinalis* protein sequences minimizes the possibility that antibody cross-reactions could take place.

To show that the polyclonal antibodies were specific for the COL-2 peptide, ELISA and competition-ELISA were carried out. The antiserum diluted up to 1:5000 exhibited maximum binding to the immobilized peptide, whereas in competitive-ELISA assay the antiserum diluted 1:3000 or 1:5000 did not react with the peptide (data not shown).

3.3. Hemocytes challenged *in vitro* with LPS express *Ci*-type IX-Col 1α-chain: flow cytometric analyses and western blotting analysis of HLS

Since the pharynx contains vessels full of hemocytes, the effect of LPS challenge on hemocytes from the hemolymph was examined *in vitro* in order to investigate their involvement in collagen production. To minimize interindividual variation and to ensure that enough hemocytes were available for all experiments, cells pooled from 20 ascidians taken from the same ascidian group were used for real-time PCR analysis of the body wall. Cells were layered (1×10^6 cells/well) onto the wells of 24-well culture plates. The cell cultures were stimulated *in vitro* with LPS (1 μg/ml) and every time 3 cultures were examined after 1, 4, and 8 h by flow cytometry with anti-*Ci*-type IX-COL2 antibodies. Every time, the results were compared with those obtained by analyzing three hemocyte cultures in the absence of LPS, and three cultures in which the primary antibodies were omitted.

Figure 3 shows a typical experiment in which challenged hemocytes express collagen. The background resulting from non-specific binding of the secondary antibody was very low and $3.9 \pm 0.4\%$ marked cells were found (Figure 3A). Unchallenged hemocytes had a basal level of collagen expression ($14.2 \pm 0.2\%$; Figure 3B), whereas the highest expression levels were reached after 1 h ($29.2 \pm 0.3\%$; Figure 3C) and after 4 h ($30.4 \pm 0.5\%$; Figure 3D) of LPS treatment and then decreased to $22.5 \pm 0.5\%$ at 8 h (Figure 3E). Values recorded at 1–8 h were significant at $P < 0.001$ when compared to untreated hemocytes.

To identify the hemocyte protein presenting *Ci*-type IX-COL2 epitopes, western blot analysis of lysate supernatant (HLS) from LPS-stimulated (4 h) and unstimulated hemocytes was carried out. The primary antibodies identified a main band at 60 kDa (Figure 3F), which was also found in HLS samples prepared from three distinct hemocyte pools obtained from 60 ascidians (20 ascidians per pool) at 4 h after the LPS injection. When the primary antibodies were previously inhibited by COL-2 peptide (competitive inhibition), no reaction was found in the western blot assay (Figure 3F). No attempts were carried out to analyze differences in density of the 60 kDa band before and after the challenge.

3.4. Hemocytes contain *Ci*-type IX-Col 1α-chain as identified by immunocytochemical reaction

To identify the hemocyte types that contained collagen, hemocytes treated *in vitro* with LPS or MS for 4 h were

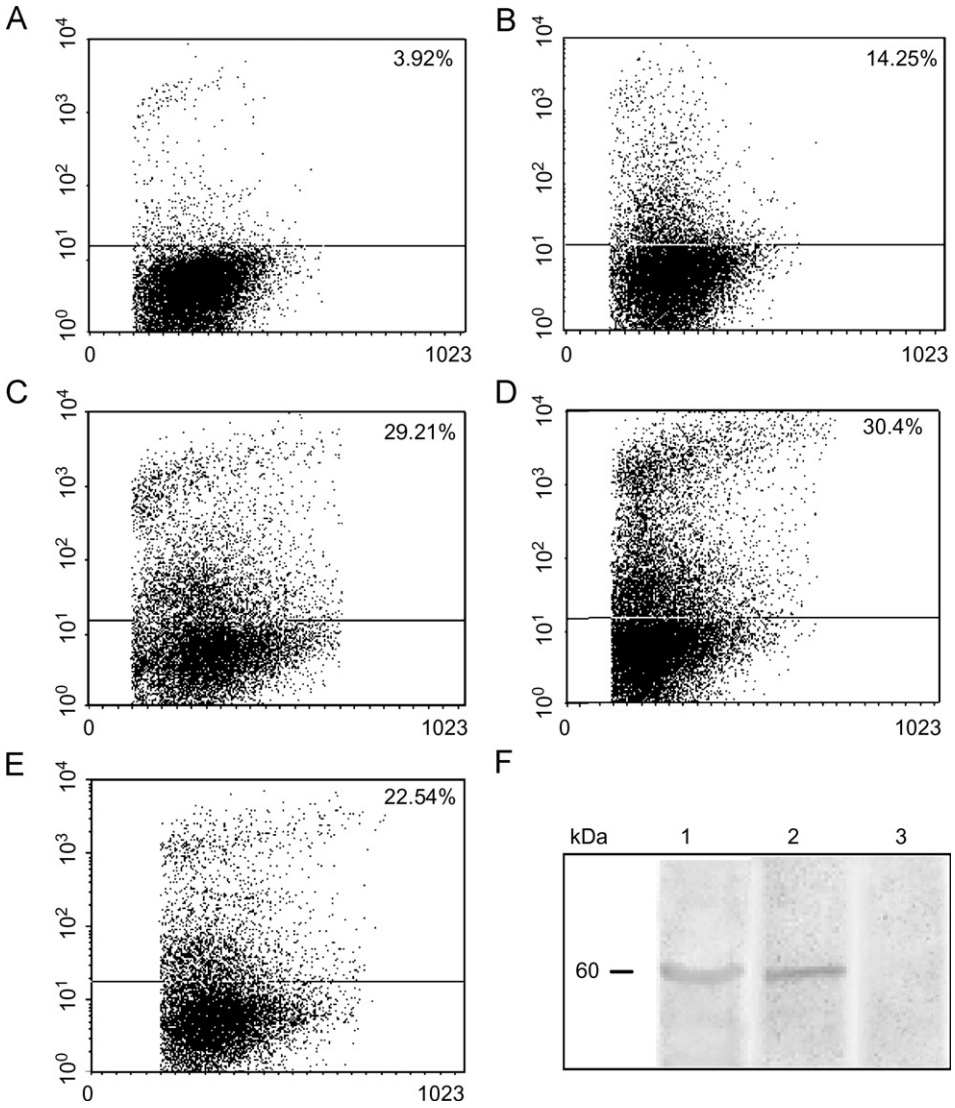

Figure 3 Flow cytometry analysis of *C. intestinalis* hemocytes maintained *in vitro* with isosmotic medium containing LPS (1 μg/ml). Anti *Ci*-type IX-collagen 1α-chain specific antibodies were used. In (A–E), a typical analysis is shown. (A) Percent of positive hemocytes after 4 h incubation with LPS and examined in the absence of primary antibodies. (B) Percent of positive hemocytes after 4 h incubation in the absence of LPS. Percent of positive hemocytes in the presence of LPS after 1 h (C), 4 h (D), and 8 h (E). (F) Western blot analysis of hemocyte lysate supernatant with anti-*Ci*-type IX-collagen (COL-2) antibodies. Lane 1: hemocytes maintained for 4 h with LPS; lane 2: untreated hemocytes; lane 3: competitive inhibition of the antiserum with the peptide COL-2. Results are presented as average values. Hemocytes were from 20 ascidians. Each assay was performed in triplicate.

examined for their immunocytochemical reaction with anti-*Ci*-type IX-COL2 antibodies. Figures 4A and B show MS or LPS-challenged hemocytes with large granules that were intensely stained (Figure 4D). In many granulocytes, only the periphery of the large granules, as well as the rim of the cytoplasm outlining the cell were stained (Figure 4E). Amoebocytes (Figure 4F) and unilocular refringent granulocytes (Figure 4G) were always negative. The specificity of the antibody reaction was demonstrated by treating the hemocytes with antiserum that inhibited the COL-2 peptide (competitive inhibition) (Figure 4C). Controls, in which the primary antibody was omitted or substituted with rabbit IgG, were negative (not shown).

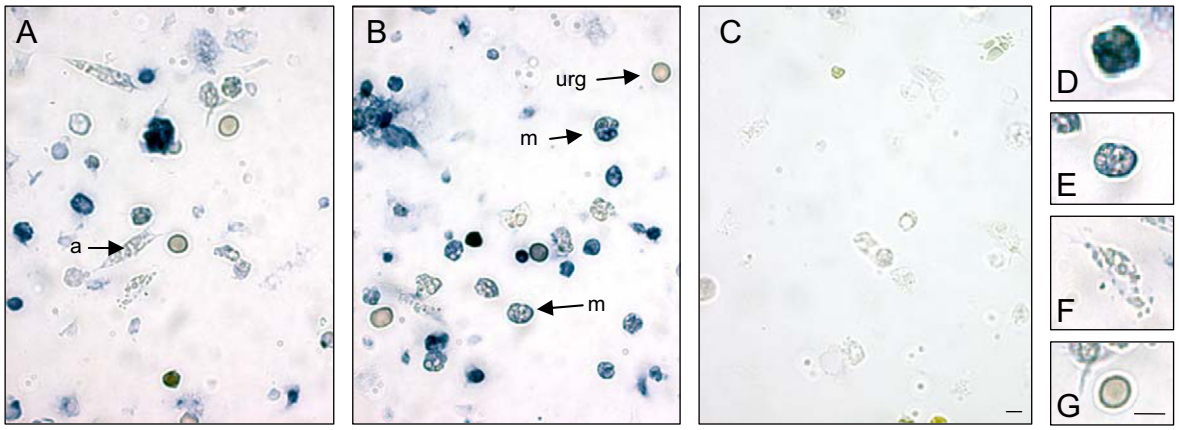

Figure 4 Immunocytochemistry of *C. intestinalis* hemocytes treated *in vitro* for 4 h with LPS. Primary antibodies: anti-*Ci*-type IX-collagen α-chain antiserum raised against the COL-2 peptide; secondary antibodies: anti-rabbit alkaline-phosphatase conjugate antibodies (m: morula cells; a: amoebocytes; URG: unilocular refractile granulocyte). (A) Hemocytes cultured in marine solution for 4 h. (B) Hemocytes cultured in the presence of LPS for 4 h. (C) Hemocytes cultured with LPS for 4 h treated with anti-Ci-type IX collagen antiserum in the presence of the peptide COL-2. (D, E) Different features of collagen-containing morula cells. (F) Amoebocyte. (G) Unilocular refractile granulocyte. Controls performed by omitting the primary antibodies or irrelevant primary antibodies (rabbit anti-goat IgGs) were negative (not shown). Bars: 10 μm.

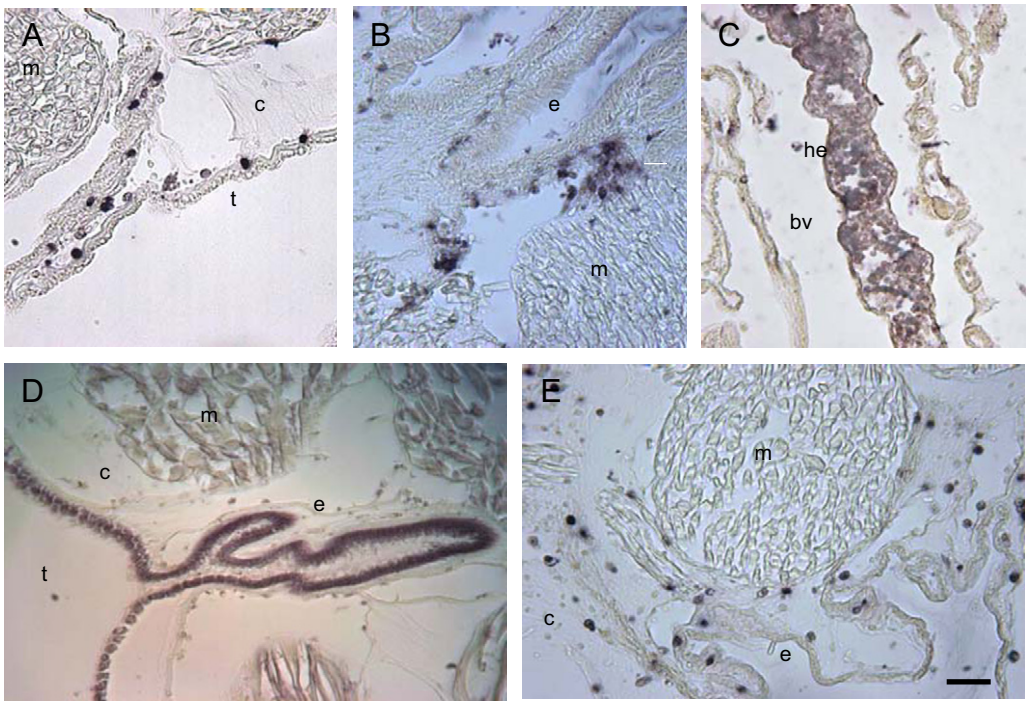

Figure 5 *In situ* hybridization with single-strand *Ci*-type IX-like collagen probe of transverse sections of *C. intestinalis* body wall fragments (t: test matrix, e: epidermis, c: connective tissue under the test, bv: blood vessel, he: hemocytes, m: muscle). (A) Marine solution-treated ascidian explant at 4 h. (B, C) LPS-treated ascidian explants at 4 h. (D) LPS-treated ascidian explant at 24 h. (E) LPS-treated ascidian explant at 192 h. Controls with the sense strand probe were always negative (not shown). Bar: 20 μm.

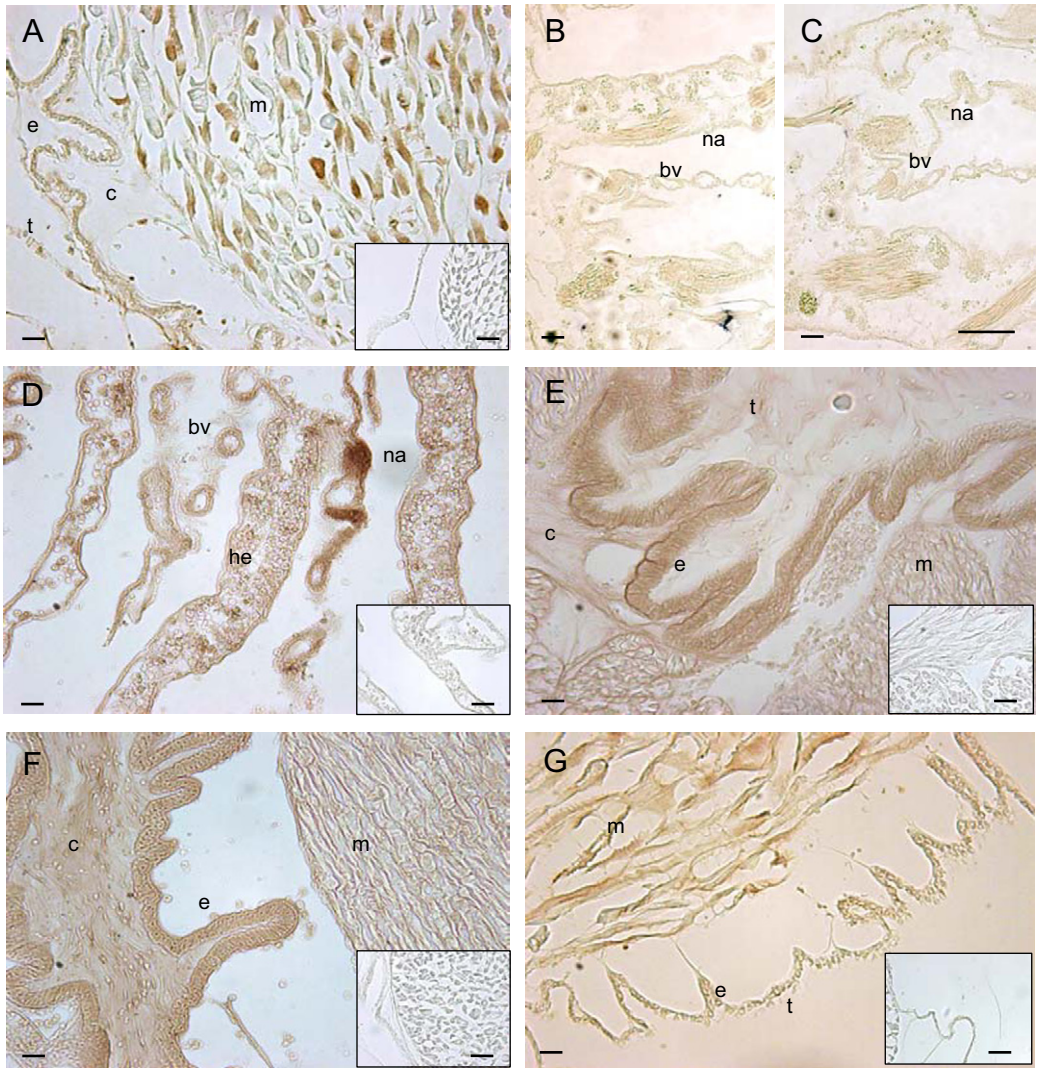

Figure 6 Immunohistochemistry of *C. intestinalis* body wall transverse sections treated with anti-*Ci*-type IX-like collagen 1α-chain primary antibodies and anti-rabbit IgG peroxidase-conjugated secondary antibodies (*t*: test matrix, e: epidermis, c: connective tissue under the test, na: nodular ansae, bv: blood vessel, he: hemocytes, m: muscle). (A) Ascidians at 4 h after marine solution injection. (B) Ascidians at 4 h after LPS injection; section treated with antiserum inhibited by COL-2 peptide. (C) Ascidians at 4 h after LPS injection; section treated with collagenase. (D, E) Ascidians at 4 h after LPS injection. (F) Ascidians at 24 h after LPS injection. (G) Ascidians at 192 h after LPS injection. Insets: control sections in which the primary antibody was omitted. Controls performed by omitting the primary antibodies or irrelevant primary antibodies were negative (not shown). Bar: 50 μm.

3.5. ISH and immunolocalization of *Ci*-type IX 1α-chain collagen in body wall injected with LPS

Histological transverse sections of body wall fragments show views from the distal to the inner side, where the following tissues can be distinguished: test matrix, epidermis, connective tissue with lacunae under the test, and pharynx blood vessels. ISH and immunohistochemistry demonstrated that both transcripts and proteins were enhanced in the inflamed tissues following LPS injection (Figures 5 and 6). Results were supported by examining tissues from three ascidians.

At 4 h after MS or LPS injection, the riboprobe was found in free cells associated with the epidermis lining the inner part of the tunic (Figure 5A, B), whereas numerous positive hemocytes could be found within the pharynx blood vessels from ascidians injected with LPS (Figure 5C). At 24 h after LPS injection, the epidermis cells appeared to be active in transcribing the gene (Figure 5D), whereas they did not show any activity at 192 h when only transcript-containing free cells were found to be associated with the epidermis (Figure 5E). In this respect, the similarity between the results presented in Figure 5E and those shown in Figure 5A (untreated ascidian) can be noted. Both of these results are in agreement with the real-time PCR analysis of the body wall (Figure 2) when the mRNA relative quantification reached the level of the untreated ascidians at 192 h. Negative controls with the sense strand probe did not show any signal (not shown).

The antibodies identified the collagen in the text matrix, epidermis, and connective tissue under the epidermis (Figure 6). Controls, carried out with competitive antiserum

inhibition of the COL-2 peptide (Figure 6B) or omitting the primary antibodies (insets in Figure 6), displayed antibody specificity. Finally, sections pre-treated with collagenase did not react with the antibodies (Figure 6C).

At 4 h after LPS injection, the pharynx blood vessel endothelium and hemocytes were outlined by the antibody reaction, while a strong reaction marked the nodular ansae (nodules with hemoblasts) of the pharynx bars (Figure 6D) and the epidermis (Figure 6E). Figure 6F shows that, at 24 h, both epidermis and tunic matrix contained collagen, whereas in Figure 6G a decreased antibody reaction was observed in the tissues.

Irrelevant primary antibodies (anti-rabbit IgG) did not show any trace of staining (not shown).

4. Discussion

In mammals, the acute inflammatory process can cause tissue injury and involves cytokine release, fibroblast proliferation, connective tissue deposition, epithelial cell migration, and collagen fiber bundle organization. Although inflammatory cells provide the initial defense against microbial invasion, tissue healing ultimately provides protection. Tissue repair begins with the arrival of fibroblasts, which form a collagen-rich granulation tissue in the wound site [31], and epidermal cells move across the injured surface. Fibrillar and non-fibrillar collagen synthesis characterizes the assembly of extracellular matrix involved in healing and the regeneration processes [31].

We have found a similar process in the body wall of the ascidian *C. intestinalis*, where several foreign substances or cells promptly induced responses involving phagocytosis, degranulation [19–21], and enhanced humoral opsonizing lectins [22]. Later, a capsule represented a reparative process with granulation tissue that, at 6 days, has been found to be rich in type-I-like collagen [23]. In this study, we showed, using real-time PCR analysis, that *Ci*-type IX-collagen (1α-chain) is promptly expressed as a component of the inflammatory response. In the inflamed tissues, the *Ci*-type IX-Col mRNA levels increased within 4 h, decreased at 8 h, and were maintained, on average, at a higher level than that observed in the untreated body wall until 192 h. Although the same response levels were displayed by four ascidians for each time point excluding individual variability, the meaning of the second increase in the collagen expression profile is unknown. However, the possibility exists that it could be due to a delayed inflammatory response by distinct tissues of the pharynx (see below). On the other hand, we do not know the regulatory effect of intracellular collagen degradation products in response to external signals, which could explain the decrease in expression of the *Ci*-type IX-collagen 1α-chain gene at 8 h. In mammals, prompt collagen degradation during inflammation and granulation tissue formation, as well as the degradation-modulating net protein production are known [32–34]. A main aspect emerging from our data is that, according to the real-time PCR analysis of body wall fragments, ISH of body wall histological sections, and hemocyte flow cytometry analysis, *C. intestinalis* hemocytes showing fibroblast-like activity appeared to be involved in this process. A type IX collagen (α-chain) was promptly expressed and reached the highest level within 4 h, when, presumably, an increased amount of hemocytes developed a fibroblast role as shown by ISH and immunohistochemical analyses. At 4 h after the injection, hemocytes inside pharynx vessels express collagen mRNA. Fibroblast-like activity of hemocytes in wound healing and around foreign material has been reported in *Limnea stagnalis* [35,36] and in the encapsulation of graft in *Planorbarius corneus* [37]. In this respect, hemocytes from the hemolymph were found to contain large granules recognized by specific antibodies. Apparently, these hemocytes are morula cells and the antigen localization at the periphery of the granules and in the cytoplasmic rim could be due to distinct hemocyte morpho-functional stages.

According to Millar [28] and De Leo et al. [29], test matrix, epidermis lining the test matrix, connective tissue close to the epidermis, and pharynx tissues with blood vessels containing hemocytes can be seen in the histological sections of the injected body wall. Likewise in mammals, the epidermis also has a role in the inflammatory response and expresses *Ci*-type IX-Col. ISH and immunohistochemical reactions carried out in tissues from three distinct ascidians showed that the epidermis lining the tunic matrix in the injection site could have a delayed activity that was mainly evident at 24 h. This observation could be in accordance with the delayed gene expression that characterized the real-time profile of the body wall response after challenge with LPS. Finally, the involvement of the epidermis is shown by ISH and immunohistochemistry analyses. With regard to collagen expression, the possibility exists that different phases characterized the response to an inflammatory stimulus, with hemocytes being the first component to be activated by LPS, which could diffuse through the tunic tissue at the injection site and promptly reached the pharynx blood vessels. In addition, the prompt inflammatory effect of the injury caused by administering LPS must be considered. Later, the epidermis appeared to be more active in expressing collagen. In accordance with our previous papers, we showed that numerous inflammatory hemocytes, including morula cells and vacuolated cells, densely populated the inflamed tunic soon after challenge, whereas a few days afterwards (3–6), the epidermis in that area presented with morphological features (larger cells with vacuoles), indicative of releasing cells [19–21]. In any case, the results reported here support that the *Ci*-type IX collagen 1α-chain is constitutively expressed in the body wall, as revealed by examining untreated ascidians and the late phase of the treated ones, whereas the inflammatory challenge enhances gene expression and collagen production.

To identify the *Ci*-type IX-collagen, polyclonal antibodies against a synthetic peptide (16 aa) designed from COL-2 (triple-helical trait) deduced amino-acid sequences of previously cloned *Ci*-type IX-Col 1α-chain were raised in rabbits [18]. The collagen nucleotide sequence was searched in the UCSC Genome Bioinformatics Site and its position was identified in chr01p:4171494–4180671 of the *C. intestinalis* genome by alignment with the BLAST-like alignment tool (BLAT, http://www.soe.ucsc.edu/~kent) program. The alignment strategy of BLAT was designed to find sequences of at least 95% similarity of 40 or more bases [38]. The COL2 peptide sequence was chosen because of the

absence of significant homologies, as shown by a BLAST search in the EMBL gene bank and *Ciona* genome sequences (JGI V2), with annotated protein sequences and by antigen-prediction programs (Sigma-Genosys) for its presumptive antigenic properties. Although epitopes are defined by stereospecific protein structure, the search of the chosen peptide sequence in *Ciona* genome sequences (JGI V2) minimizes the possibility that antibody cross-reactions can take place. In addition, the specificity of antibodies for the COL2 peptide was shown by ELISA and competition ELISA, in which antibody binding to immobilized peptide was inhibited by COL2 peptide in solution; furthermore, the competitive inhibition was effective in blocking the antibody reaction in an immunoblotting assay of the hemocyte lysate supernatant. In addition, the specific binding of antibodies with *Ci*-type IX-collagen in histological sections was shown by treating the sections with collagenase that decreased the antibody reaction. In any case, the consistent expression levels observed throughout the time course by body wall explants analyzed by real-time PCR indicated that the collagen identified by the antibodies could be the *Ci*-type IX-Col 1α-chain.

Although we were not able to distinguish if epithelial cells or hemocytes were the migrating cells, the presence of riboprobe-marked cells associated with the epidermis suggested that cells move to form a collagen-rich granulation tissue at the inflamed site. At 4–8 h, the epidermis mainly contained protein, then (24 h) both collagen mRNA and protein were expressed in the epidermis, but were decreased in the body wall by 192 h after stimulation, when the expression level was similar to that of the untreated ascidians. In addition, antibodies identified collagen associated with the epidermis proximal border and vessel endothelium in accordance with the fine structure finding reported in a previous paper [29], in which basement membrane adherent to the epidermis and vessel endothelium was shown. The possibility exists that *Ci*-type IX-Col may be associated with a Type-I-like collagen previously identified by heterologous antibodies in the inflamed tissue [23].

Finally, western blot analysis with specific antibodies was carried out to verify the presence of a protein component with Ci-type IX-COL2 epitopes in hemocytes. The immunoblot displayed a main FACIT collagen component approximately 60 kDa in size that was found in hemocyte lysate supernatant examined before and after (4 h) an *in vitro* or *in vivo* LPS challenge. Competitive inhibition of the antiserum with the peptide COL-2, as an antigen, further demonstrated the specificity of the antibodies, while the apparent molecular size of this band is in conformity with the already known apparent molecular size of Ci-type IX-Col deduced from the amino-acid sequence previously reported by Vizzini et al. [18]. Differences in band density were not analyzed and research is in progress to evaluate the possible density variation of this band in LPS-treated hemocytes compared to untreated ones. On the other hand, intracellular degradation of collagen molecules could be expected during an inflammatory process to produce pro-inflammatory fragments [32–34].

According to a previous paper [22], the *C. intestinalis* inflammatory response to LPS challenge appears to be composed of a complex reaction that includes inducible lectins as opsonins, and FACIT-collagen enhancement (present paper) that participates in granulation phase and wound healing.

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