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In Vitro Synthesis of Proteoglycans and Collagen in Primary Cultures of Mantle Cells from the Nacreous Mollusk, *Haliotis tuberculata:* A New Model for Study of Molluscan Extracellular Matrix

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Abstract: In Mollusca, the mantle produces an organic matrix that mineralizes in time to make shell. Primary mantle cell cultures from the nacreous gastropod *Haliotis tuberculata* have been established as useful experimental model to investigate in vitro synthesis of both proteoglycans/glycosaminoglycans (PGs/GAGs) and collagen. First, we tested different enzymatic digestion procedures to find the method that gives the highest percentage of viable and adherent cultured cells. Enzymatic digestion with 0.1% pronase plus 0.1% collagenase was routinely used. Six days after the initiation of culture, about 80% of cells were viable, among which 20% were adherent as quantified by the MTT reduction assay. In addition, the protein synthesis estimated by [^{3}H]leucine incorporation remained constant during this period. For the first time, we demonstrated a de novo synthesis of PGs/GAGs and collagen in primary cultures of mantle cells. After 48 hours of labeling, among the [^{3}H]-D-glucosamine macromolecules synthesized, [^{3}H]PGs/GAGs represented 43%, divided into 45% heparan sulfate, 37% chondroitin/dermatan sulfate, and 6% hyaluronic acid. Early elution on anion-exchange chromatography of these PGs/GAGs indicated that most of them appeared as undersulfated GAG molecules. De novo synthesis of collagen represents 4.52% ± 0.84% (SD) with respect to the total protein synthesis. Such a model will facilitate studies on the synthesis of PGs/GAGs and collagen as components of the extracellular matrix and its regulation in Mollusca. Both PGs/GAGs and collagen participate in molecular events that regulate cell adhesion, migration, and proliferation. Further studies with this type of in vitro model should provide knowledge about novel aspects of molluscan cell signaling, in relation to extracellular matrix components.

Key words: Mollusca, mantle cells, proteoglycans, collagen.

INTRODUCTION

In multicellular organisms, the extracellular matrix (ECM) maintains and defines the shapes of tissues and organs. In addition to its structural role, ECM participates in molecular events that regulate cell adhesion, migration, and proliferation (Har-El and Tanzer, 1993; Taipale and Keski-Oja, 1997; Wight et al., 1992). In invertebrates as well as vertebrates, ECM is composed mainly of collagens and proteoglycans (PGs) grouped into several different families (Har-El and Tanzer, 1993). Collagen molecules represent a het-

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erogeneous group of proteins. More than 19 different collagen types have been described in vertebrates, each with characteristic tissue distribution and function, and a comparable heterogeneity seems to exist in invertebrates (Kingston, 1991; Kramer, 1994; Weckmann and Cabral, 1996; Engel, 1997). Proteoglycans are complex sulfated macromolecules composed of linear chains of glycosaminoglycan (GAG) covalently attached to a core protein. Glycosaminoglycans belong to a family of closely related molecules, which includes hyaluronic acid (HA), heparin (H), chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS). Hyaluronic acid and heparin differ from other GAGs in that they appear as free polysaccharide chains (Hardinghan and Fosang, 1992). PGs/GAGs have been most extensively studied in vertebrates, but have also been detected in invertebrates (Har-El and Tanzer, 1993). Thus collagen and PGs/GAGs appear to be ubiquitous components of vertebrate as well as invertebrate tissues and are widely distributed in mollusks (Nader et al., 1996).

The use of in vitro systems and in particular primary culture methodology has opened up new approaches, in vertebrates, for studying ECM synthesis, as well as the regulation of synthesis, and the role of the ECM in the control of cellular processes. In marine mollusks, many attempts have been made to establish long-term primary cell cultures; however, little progress has been made. Nevertheless, some suitable models have been used successfully to investigate specific processes: for example, cultured amebocytes from pearly oyster, cultivated alone or in coculture with mantle cells, to analyze the wound-healing process (Awaji and Suzuki, 1998; Suzuki et al., 1991); hemocytes from *Haliotis tuberculata* to study effects of vertebrate growth factors on molluscan cell metabolism (Lebel et al., 1996); gill cells from the butterfish clam for pathological studies (Auzoux et al., 1993); larval cells from *Haliotis rufescens* for analysis of myogenesis (Naganuma et al., 1994); and mussel digestive gland cells for environmental toxicology studies (Robledo and Cajaraville, 1997).

In Mollusca, mantle cells are known to produce and secrete an organic matrix that mineralizes in time to make shell (Simkiss and Wilbur, 1989). Moreover, PGs/GAGs such as HS-PGs have been detected in the mantle from the mollusk *Helix aspersa* and from the freshwater mussel *Anodonta californiensis* (Hovingh and Linker, 1993, 1998). In the same way, Yoneda et al. (1999) have demonstrated the presence of collagen transcript in material extracted from mantle cells of the abalone *Haliotis discus.* Given that collagen and PGs/GAGs have been identified in mantle from mollusks, it seems likely that mantle cells could provide a suitable model to investigate the in vitro synthesis of ECM by molluscan cells.

The aim of the present study was to establish a suitable in vitro model from primary cultures of mantle cells from *Haliotis tuberculata,* a nacreous mollusk, and subsequently to investigate the in vitro synthesis of ECM components. In the first part of this study, we tested different enzymatic digestion procedures in order to choose the method giving the highest percentage of viable and adherent cultured cells. During the first 6 days of culture, mantle cell viability and metabolism were tested. In the second part of this study we investigated the ability of cultured mantle cells to synthesize the main typical PG/GAG members and collagen as components of ECM.

Materials and Methods

Primary Cell Cultures

Adult abalones, *Haliotis tuberculata,* 6 to 8 cm in shell length, were purchased from an aquacole farm on the West Coast of the Cotentin peninsula (Manche, France) and acclimated to laboratory conditions for at least 2 weeks before experimentation. Animals were maintained in natural and continuously aerated seawater at seasonal ambient temperature. They were fed daily with a mixed algal diet. The whole mantles were removed and washed (2 × 90 minutes) with sterile Hanks'-199 medium modified by addition of 250 mM NaCl; 10 mM KCl; 25 mM $MgSO_4$; 2.5 mM $CaCl_2$; 10 mM Hepes, final pH 7.4, 1000 mOsm/L, and supplemented with antifungal and antibacterial substances (streptomycin sulfate, 100 μg/ml; penicillin G, 60 μg/ml; polymyxin B sulfate, 35 μg/ml; bacitracin, 70 μg/ml; amphotericin B, 0.20 μg/ml; and nystatin, 8 μg/ml), and with L-glutamine (2 mM). Decontaminated tissues were rinsed once with modified Hanks'-199 medium. This step and subsequent steps were performed with the modified medium supplemented only with streptomycin sulfate, 100 μg/ml; penicillin G, 60 μg/ml; and L-glutamine (2 mM).

Then, mantle was fragmented into small pieces and placed in one of the following dissociation media (approximately 3 ml/g tissue): (A) 0.1% pronase (EC 3.4.24.31) plus 0.1% collagenase (EC 3.4.24.3); (B) 0.025% hyaluronidase (EC 3.2.1.35); (C) 0.1% pronase, 0.1% collagenase plus 0.01% hyaluronidase, or (D) 0.01% pronase plus 0.01% collagenase. Digestions were carried out at 15°C for 90 min-

utes (2 times) for A, B, and C, or at 6°C overnight for D, with continuous and gentle stirring. After digestion, dissociated cells were collected by filtration through 100-μm nylon meshes and centrifuged at 4°C for 15 minutes at 280 *g*. The resulting pellet was washed twice in modified Hanks'-199 medium and finally resuspended in 8 ml. Cells were kept on ice. This procedure was repeated once with the nondigested tissue recovered on nylon meshes, and finally dissociated cells were pooled.

Immediately after dissociation, cells were counted with a hemocytometer, and cell viability was estimated by dye exclusion in a 0.2% trypan blue solution. Then, cells were plated and maintained at 15°C in a humidified incubator (CO_2-free) in darkness. For cultures maintained longer than 3 days, fresh medium (50% of the initial volume) was added once to each well. In some experiments, effects of several adhesines were tested on cell adhesion 6 days after the initiation of culture. Thus, six-well culture plates were coated with fibronectin (Sigma; F 3667, 6 μg/cm^2), poly-L-lysine (Sigma; P 4707, 120 μg/cm^2), and ECM gel (Sigma; E 1270, 100 μg/cm^2) according to the manufacturer's instructions.

After initiation of the culture, the viability of cells was also determined by the MTT reduction assay (Mosmann, 1983) adapted to molluscan cells (Naganuma et al., 1994; Lebel et al., 1996; Sud et al., 1998). For assays, MTT solution was added to each dish to be tested (0.5 mg MTT/ml in final concentration). Reaction was stopped by addition of an equal volume of isopropanol containing 0.04 N HCl. The plates were shaken at room temperature, and absorbance was measured at wavelength of 570 nm with a reference of 630 nm.

Protein Synthesis

Cells were plated at 10^6 cells per well in six-well culture plates. Twenty-four hours after the beginning of culture, the volume of medium was adjusted at 1.2 ml per dish. L-[3,4,5-^{3}H]-Leucine (sp act, 180 Ci/mmol; NEN Life Science Products) was added to each dish (1 μCi per dish), and cultures were then performed at 15°C for 24 hours. Incorporation of L-[3,4,5-^{3}H]-leucine in cells was determined as previously described (Lebel et al., 1996).

Proteoglycans and Glycosaminoglycans Syntheses

Extraction, determination, and repartition of PGs and GAGs were based on a typical protocol that was previously described (Thiébot et al., 1997). The criterion for identification of PGs/GAGs was the modification of the k_{av} value as determined by gel filtration mobility of the molecules subjected to specific digestions. Cells were plated at 12×10^6 cells per 75-cm^2 culture flask, and synthesis of PGs and GAGs was analyzed 24 hours after the beginning of culture. The pulsing medium containing 4 μl/ml (1 μCi/μl) of [6-^{3}H]-D-glucosamine (sp act, 20–45 Ci/mmol; NEN Life Science Products), diluted in culture medium, was added to each flask, and cultures were maintained at 15°C for 48 hours. In this protocol, the whole PG/GAG synthesis (cell layer plus medium-neosynthesized material) was considered without any distinction. Then, in order to achieve extraction, cultures were maintained for 6 hours in the presence of an equal volume of TUT (2×) at 4°C (TUT buffer: 50 mM Tris, pH 7.2, containing 4 M urea, 0.5% Triton X-100, 0.02% NaN_3, and a mixture of protase inhibitors, 10 mM EDTA, 100 mM 6-aminohexanoic acid, and 0.2 mM phenylmethyl sulfonylfluoride). Labeled PGs/GAGs both in the cell fraction and released into the culture medium were first purified by anion-exchange chromatography (DEAE-Trisacryl). Samples were dialyzed against TUT buffer and applied to the DEAE-Trisacryl column (1 × 5 cm) previously equilibrated with TUT buffer. Elution was performed with 50 ml of TUT buffer, then with a linear gradient of NaCl (0 to 1.2 M) in the same buffer. A flow rate of 40 ml/h was used, and 1-ml fractions were collected. Radioactivity-rich fractions expected to contain the peak of PGs and GAGs (eluted between 0.1 and 0.3 M NaCl) were pooled and precipitated with ethanol. The pellet was redissolved in distilled water, aliquoted, and lyophilized, and samples were reconstituted with different buffers depending on subsequent treatments.

An aliquot was dissolved in TUT buffer, cleared by centrifugation (10,000 *g* for 10 minutes), and then submitted to gel filtration on a Superose 6 column (1 × 30 cm). The elution was carried out with 30 ml of TUT buffer at a flow rate of 15 ml/h, and 0.4-ml fractions were collected. A second aliquot was then submitted to 0.1 M NaOH in the presence of 1 M $NaBH_4$ in order to induce β-elimination (Carlson, 1968). After 72 hours of incubation at 45°C, the reaction was stopped by neutralization with glacial acetic acid. In parallel, PG-specific digestions were performed. HS-PGs were degraded by deaminative cleavage using the pH 1.5/HNO_2 method (Conrad et al., 1977). CS/DS-PGs were digested with chondroitinase ABC (EC 4.2.2.4, 1 IU/ml) in 50 mM Tris-HCl buffer (pH 8.0) containing 60 mM sodium acetate, 50 mM NaCl, 10 mM NaF, 0.1 mg/ml bo-

vine serum albumin (BSA) (Kleine and Merten, 1981). Hyaluronic acid was degraded by *Streptomyces* hyaluronidase (EC 4.2.2.1, 3 IU/ml) in 0.02 M acetate buffer, pH 5, for 24 hours at 37°C (Ohya and Kaneko, 1970). In all cases, the extent of degradation of polysaccharides was determined by Superose 6 chromatography and the radioactivity was counted after addition of 4 ml of liquid scintillation to each vial.

Collagen Synthesis

Collagen synthesis was based on the protocol developed by Peterkofsky and Diegelmann (1971). As has been done for PGs/GAGs, the whole collagen synthesis (cell layer plus medium-neosynthesized material) was considered without any distinction. Cells were plated at 4×10^6 cells per well in six-well culture plates, and synthesis of collagen was analyzed after the beginning of culture. The pulsing medium containing fresh ascorbate (300 μM), β-aminopropionitrile (200 μM), and 1.5 μl (1 μCi/μl) of L-[2,3,4,5-^{3}H]-proline (sp act, 80 Ci/mmol; NEN Life Science Products) was added to each well, and cultures were performed at 15°C for 48 hours. After incubation, cells were scraped and transferred with the culture medium in tubes that were centrifuged at 5000 *g* for 20 minutes at 4°C. Pellets were sonicated in 1 ml of 50 mM Tris-HCl, pH 7.6, containing 10 mM $CaCl_2$. Then, the lysate was cleared by centrifugation (500 *g* for 20 minutes at 4°C), and the supernatant was placed in two tubes (400 μl per tube). One tube received an additional 20 μl of $CaCl_2$-TES buffer and the other 20 μl (1 U/μl) of *Clostridium histolyticum* collagenase (Worthington, CLSPA, 3.4.24.3) in $CaCl_2$-TES buffer. The tubes were incubated at 37°C for 17 hours.

The reaction was stopped on ice, and proteins were precipitated by adding trichloroacetic acid (final concentration, 5%) containing tannic acid (final concentration, 0.25%) in the presence of BSA. The tubes were chilled on ice for 10 minutes and centrifuged at 4°C for 10 minutes at 5000 *g*. The supernatant was discarded, and the pellets were washed three times with 10% trichloacetic acid containing 0.25% tannic acid. Then, 250 μl of NaOH (1 M) was added in order to dissolve the pellet, and 200 μl was transferred in a scintillation vial containing 200 μl HCl (1 M). The radioactivity was counted after addition of 4 ml of liquid scintillation to each vial. Blank controls were performed with cells that were precipitated with trichloroacetic acid prior the addition of [^{3}H]proline. Collagen proteins (*CP*) were determined from the difference in radioactivity incorporated into total proteins (*TP*) and the noncollagenous proteins (*NCP*). The percentage of collagen was calculated according to Peterkofsky and Diegelmann (1971) using the equation, % Collagen = dpm (*CP*)/[dpm (*CP*) + 5,4 dpm (*NCP*)], to correct for the high proline content of collagen compared with noncollagen proteins.

Statistical Analysis

Significance of the difference between mean values was estimated using Student's *t* test. Each experiment was repeated at least three times, and for one experiment the means were calculated from triplicates.

Results

Cell Cultures

To dissociate mantle cells, four different enzymatic treatments were tested. Immediately after the dissociation, the number of cells and the percentage of viable cells (based on the trypan blue exclusion staining test) were determined. As shown in Figure 1, the highest number of isolated cells per gram of fresh tissue was obtained after digestion by hyaluronidase 0.025% (2 × 90 minutes) (66×10^6 cells/g of mantle). In this case, the number of viable cells was 36×10^6 cells/g, which represents 55% of viable cells. In fact, the highest percentage of viable cells (81%) was obtained after digestion with protease-collagenase (0.1%-0.1%). The two others enzymatic treatments (C or D) gave similar numbers of viable cells, about 20×10^6 cells/g of mantle (Figure 1).

The number of viable cultured cells was also determined using the MTT reduction assay. The absorbance was directly proportional to the number of cultured cells per dish. This linear function ($y = 0.0826 \times -0.0024$, $r^2 = .999$) included the greater value tested, 2×10^6 cells per dish (data not shown). One day after initiation of the culture, the viability of cells was evaluated by the MTT test, and this value was compared with the one obtained from the trypan blue exclusion assay conducted immediately after dissociation (Figure 2). For the protease 0.1% plus collagenase 0.1% digestion (2 × 90 minutes), approximately 100% of cells were viable (including 55% of adherent cells) 1 day after initiation of the culture (Figure 2, a), and 77% (including 19% of adherent cells) after 6 days (Figure 2, b). Other dissociation procedures (B, C, and D) gave significantly different results, especially with regard to the number of viable adherent cells, 1 day and 6 days after the beginning of

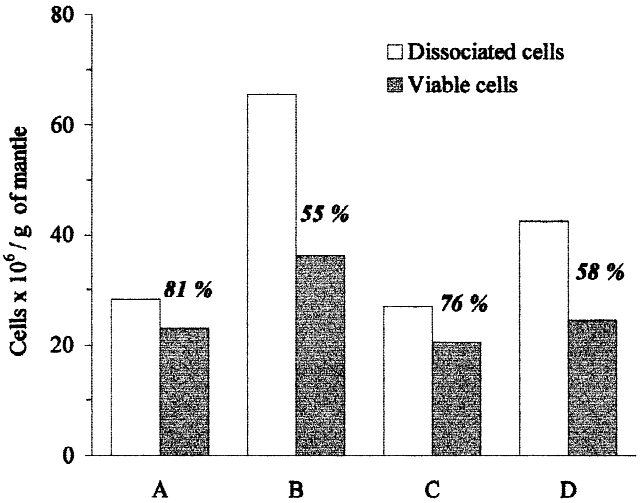

Figure 1. Cell number and viability, as tested by the trypan blue exclusion test, obtained per gram of mantle using the four enzymatic dissociation procedures described in "Materials and Methods." (A) 0.1% pronase plus 0.1% collagenase; (B) 0.025% hyaluronidase; (C) 0.1% pronase, 0.1% collagenase plus 0.01% hyaluronidase; (D) 0.01% pronase plus 0.01% collagenase. Dissociation procedures were conducted at 15°C for 90 minutes (2 times) for A, B, and C and at 6°C overnight for D. *Italic values* indicate the percentage of viable cells in comparison with total dissociated cells.

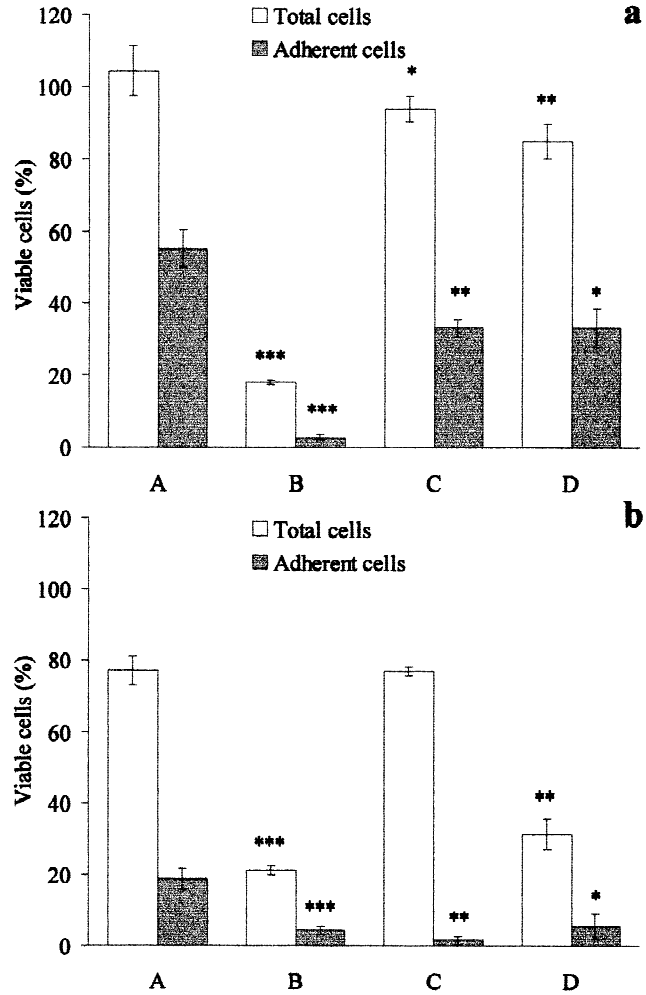

Figure 2. Effects of enzymatic dissociation procedures on cell viability and attachment (as determined by the MTT reduction assay) 1-day (**a**) and 6-days (**b**) after the initiation of cultures. Mantle cells were dissociated and plated at a density of 10^6 cells per well in six-well culture plates and cultured at 15°C in modified Hanks'-199 medium. The MTT assay was performed as described in "Materials and Methods." Each data point (OD 570 nm − ref. 630 nm) represents the mean ± SD of triplicate cultures. Results were expressed as percentages of viable plated cells as determined by the trypan blue exclusion test. Significant difference from procedure A at $P < .05$ (*); $P < .01$ (**); $P < .001$ (***).

culture. At the end of the culture (6th day), the percentage of viable adherent cells was 4% for B, 2% for C, and 6% for D, in comparison with 19% for A (Figure 2). Thus, given the rate of cell viability and adherence, procedure A (protease-collagenase, 0.1%-0.1%) was routinely used.

The viability of mantle cells as well as [^{3}H]leucine incorporation (Figure 3) remained constant during the first 6 days of culture. However, a significantly higher value was registered for the MTT assay 2 days after the initiation of cultures.

In culture dishes, cells in suspension appeared as isolated cells or more frequently as aggregated cells (Figure 4). On the basis of their morphological characteristics, floating cells appeared as epithelial-like cells. Part of the cells adhered to the surface of dishes; they began to flatten after initiation of the culture and formed a monolayer. Adherent cells are similar to epithelial-like cells and fibroblast-like cells.

In order to increase their adherence rate, cells were cultured in dishes coated with ECM gel (100 μg/cm^2), poly-L-lysine (120 μg/cm^2) or fibronectin (6 μg/cm^2). After 6 days of culture, in spite of a small increase in the percentage of adherent cells for the poly-L-lysine coating, no significant result was registered (Figure 5).

Proteoglycans/Glycosaminoglycans Syntheses

Forty-eight hours after labeling with [^{3}H]-D-glucosamine, material (cell layer plus medium material) was purified by

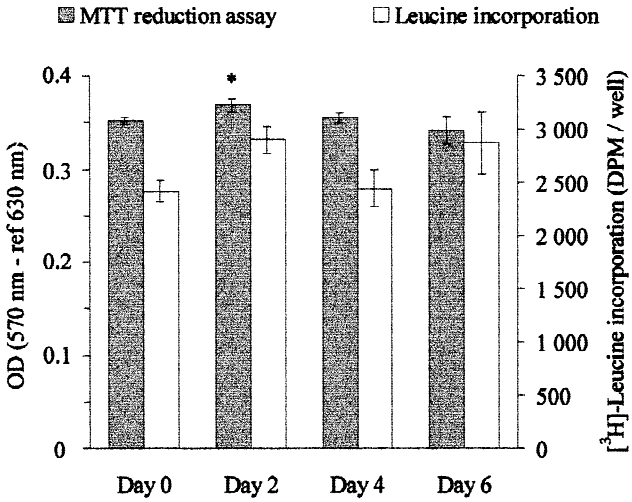

Figure 3. Incorporation of [^{3}H]leucine in relation to viability of mantle cells during the first 6 days of culture. Cells were dissociated (procedure A, 0.1% pronase plus 0.1% collagenase), seeded at 10^6 cells per well in six-well culture plates, and cultured at 15°C in modified Hanks'-199 medium. [^{3}H]leucine was added to the wells 24 hours before the measurement of [^{3}H]leucine incorporation. Others wells were seeded at the same concentration of cells, and the MTT assay was performed as described above. Each data point represents the mean ± SD of triplicate cultures. Significant difference from control cells (day 0) at $P < .05$ (*).

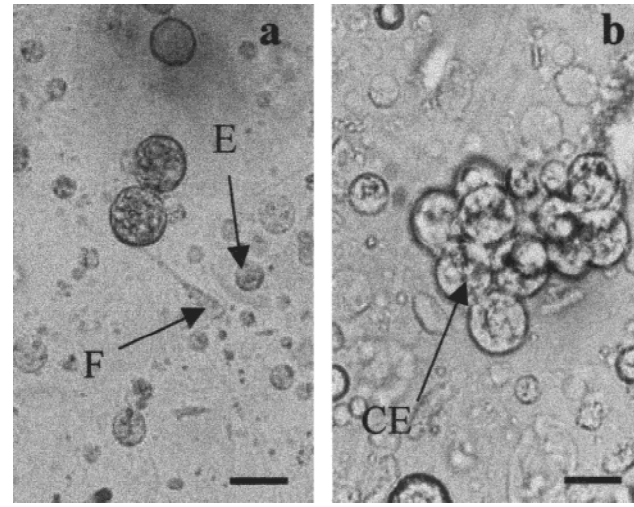

Figure 4. Light microscopic pictures showing morphology of 2-day-old primary cultures of mantle cells. Adherent cells (**a**) were mostly fibroblast-like cells (*F*) and to a lesser extent epithelial-like cells (*E*). Floating cells (**b**) appeared to be mostly composed of clusters of epithelial-like cells (*CE*). Scale bar = 20 μm.

ion-exchange chromatography (Figure 6). The elution profile revealed two major peaks closely eluted at low salt concentration (about 0.1–0.2 M NaCl), showing that these molecules were weakly polyanionic. This material was submitted to Superose-6 gel filtration chromatography (Figure 7). The untreated sample contained three major [^{3}H]glycosamine components. The first peak (I) was recovered in the void volume, the second (II) was eluted with a k_{av} value between 0.4 and 0.7, and the third (III) was eluted with a k_{av} value of 0.9 (Figure 7). In parallel, the digestion of [^{3}H]macromolecules by *Streptomyces* hyaluronidase showed that 6% of labeled material shifted as hyaluronic acid as it was eluted, after digestion, with a peak k_{av} value of 0.8 (Figure 8). To determine whether the [^{3}H]macromolecules synthesized by mantle cells were of proteoglycan nature, material was first subjected to alkali treatment, which led to the release of intact [^{3}H]glycosaminoglycan chains from [^{3}H]proteoglycans. The elution profile of the material eluted with a k_{av} value inferior to 0.7 (peaks I and II) changed such that about 40% of the radioactivity was translated to a k_{av} value of 0.9, corresponding to GAGs-containing fractions (Figure 7). To determine the nature of PGs, samples purified by ion-exchange chromatography were subjected respectively to chondroitinase-ABC and HNO_2 treatments and then to Superose-6 gel filtration chromatography. Of these recovered GAGs, 37% were degraded with the enzyme (CS/DS-PGs) and 45% were depolymerized after HNO_2 treatment at pH 1.5 (HS-PGs) (Figure 8). In addition, 18% of alkali-sensitive material remained intact after chondroitinase-ABC or HNO_2 treatment (Figure 8).

Collagen Synthesis

To determine whether mantle cells were synthesizing collagen, cultured cells were labeled with [^{3}H]proline for the first 48 hours. Then, the material corresponding to both cell layer and medium material was tested for the presence of collagenase-sensitive proteins. The highly purified collagenase used was found to be free of nonspecific proteinase activity (Diegelmann et al., 1990). Figure 9 shows the results obtained from a typical experiment of collagen synthesis. In cultured mantle cells, de novo synthesis of collagen, calculated according to Peterkofsky and Diegelmann (1971), represents 4.52% ± 0.84% (SD) of total protein synthesis.

Discussion

In multicellular organisms, ECM is composed mainly of collagens and proteoglycans. In Mollusca, the presence of

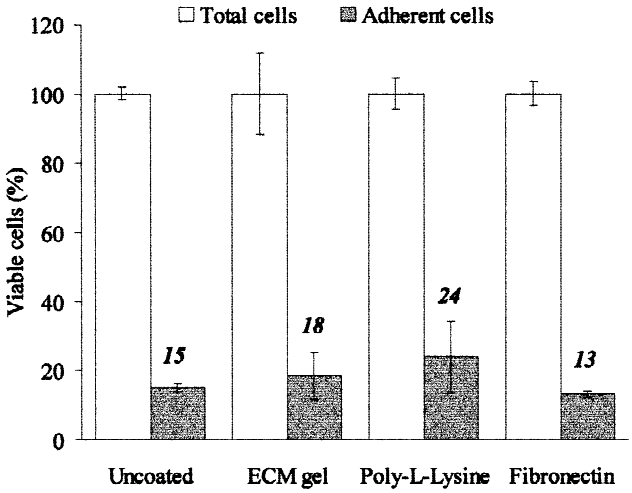

Figure 5. Influence of various extracellular matrices on the attachment of mantle cells in culture. Six-well culture plates were coated with ECM gel (100 μg/cm^2), poly-L-lysine (120 μg/cm^2), or fibronecitn (6 μg/cm^2). Mantle cells were dissociated (procedure A, 0.1% pronase plus 0.1% collagenase), seeded at a density of 10^6 cells per well, and cultured at 15°C in modified Hanks'-199 medium. Then the MTT test was performed during the first 6 days of cultures. *Italic values* indicate the percentage of adherent cells in plastic control wells or in well coated with adhesives. Each data point (OD 570 nm − ref. 630 nm) represents the mean ± SD of triplicate cultures.

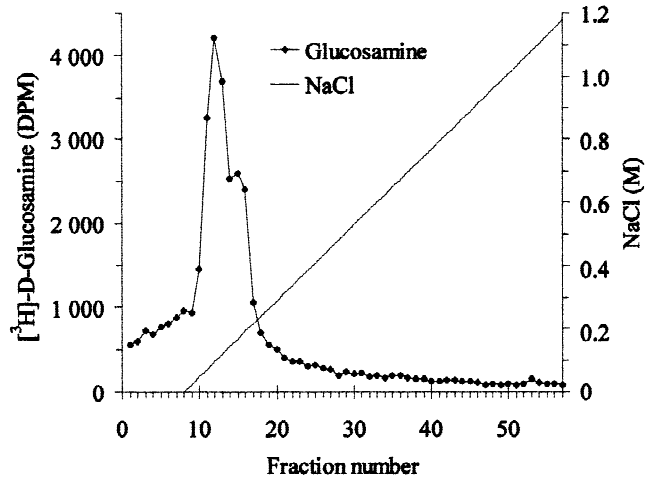

Figure 6. Anion-exchange chromatography of extract of mantle cells cultured in presence of [^{3}H]-D-glucosamine. Cells were dissociated (procedure A, 0.1% pronase plus 0.1% collagenase), plated at 12 × 10^6 cells per 75-cm^2 culture flask, and cultured at 15°C for 48 hours; 24 hours after the beginning of culture [^{3}H]D-glucosamine (1 μCi/μl) was added for the last 48 hours of culture. Thereafter medium plus cell layer TUT-extracted fractions pooled from eight flasks were applied to a DEAE-Trisacryl column and eluted with a 0–1.2 M NaCl linear gradient as described in "Materials and Methods." A typical experiment was repeated at least three times.

collagen molecules has been demonstrated for several years, in particular, mussel byssus collagen from bivalves, squid skin collagen, or abalone *Haliotis* muscle collagen (Gosline and Shadwick, 1983; Kimura and Tanaka, 1983; Olaecha et al., 1993; Qin and Waite, 1995; Deming, 1999; Yoneda et al., 1999). In the same way, PGs appear to be widely distributed in invertebrates, and chondroitin sulfate as well as heparan sulfate have been detected in mollusks (Cassaro and Dietrich, 1977; Dietrich et al., 1983; Nader et al., 1984, 1996; Brandan et al., 1992; Oliveira et al., 1994, Hovingh and Linker, 1998; Volpi et al., 1998). The shell growth begins with the secretion by the mantle cells of an ECM, which is most responsible for later events leading to the mineralization process (Simkiss and Wilbur, 1989). The present study describes a useful in vitro model from primary cultures of mantle cells from *Haliotis tuberculata* and, for the first time, evidence for the ability of these cultured cells to synthesize in vitro PGs/GAGs and collagen as components of the ECM.

First, our main interest was to obtain the highest number of viable and adherent mantle cells. For this reason, the protease 0.1% plus collagenase 0.1% (2 × 90 minutes) dissociation was chosen. Routinely, 20,000 cells per gram of fresh tissue were obtained, and the cell viability remained very high (80%) until 6 days after the initiation of the culture, with a rate of adherent cells of about 20% at this time. Physical manipulation or enzymatic treatment may produce cells with greatly decreased viability. Results registered with the protease 0.1%–collagenase 0.1% digestion demonstrated that most cells had undergone little damage during this dissociation procedure. Primary cultures of mantle cells provided the two main cellular categories previously reported in molluscan cell cultures by several authors: fibroblast-like and epithelial-like cells (Auzoux et al., 1993; Naganuma et al., 1994; Odintsova et al., 1994; Takeuchi et al., 1994; Lebel et al., 1996). Since cell attachment facilitates long-term cultivation, we have attempted to enhance cell adhesion using suitable substrate. Three adhesive factors were tested to assess their effects on cell attachment: polylysine, fibronectin, and ECM gel. In wells coated with poly-L-lysine, a very small increase in cell attachment was observed, but the difference in comparison with control wells was not statistically significant. To date, the effect of substrate on molluscan cell adhesion remains controversial. Odintsova et al. (1994) have shown that some adhesion

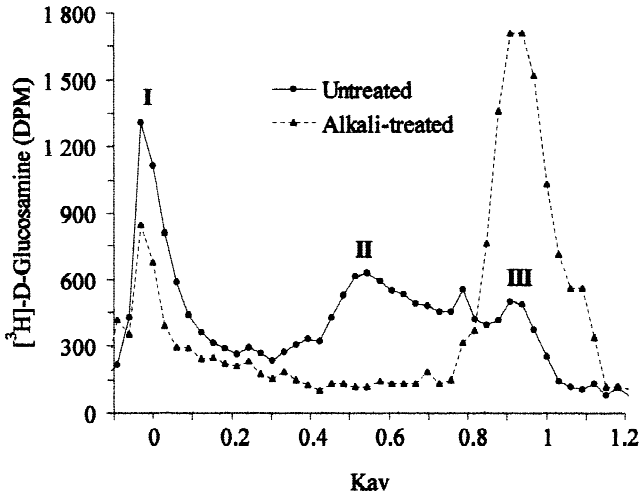

Figure 7. Typical gel filtration chromatogram of DEAE-Trisacryl purified peaks (Figure 6) on Superose-6. Proteoglycans were purified in each case using DEAE-Trisacryl column (Figure 6) and then subjected to a Superose-6 column before (*solid line*) or after alkali treatment (*broken line*). Aliquots of the same material (Figure 6) were also applied to the column after C-ABC treatment and after HNO_2 treatment, as described in "Materials and Methods." The patterns of elution profiles were similar (data not shown). A typical experiment was repeated at least three times.

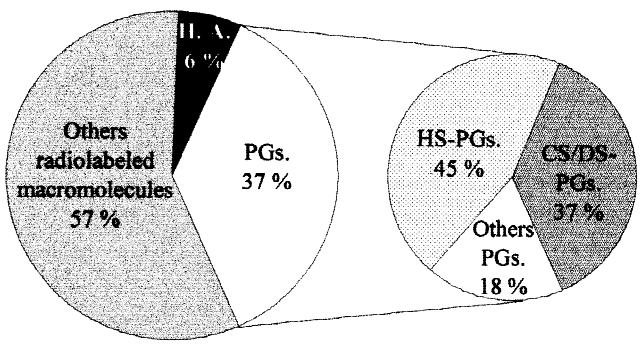

Figure 8. Distribution of [^{3}H]glycosamine-radiolabeled molecules in mantle cell cultures. Specific treatments (Figure 7) revealed the rate of the following labeled molecules. First, values are expressed as a percentage of PGs (proteoglycans), H.A (the hyaluronic acid GAG), and other radiolabeled glycoproteins. Second, the distribution of PGs is expressed in percentage of HS-PGs (heparan sulfate proteoglycans) and CS/DS-PGs (chondroitin sulfate and dermatan sulfate proteoglycans). Each value represents the mean of three separate experiments.

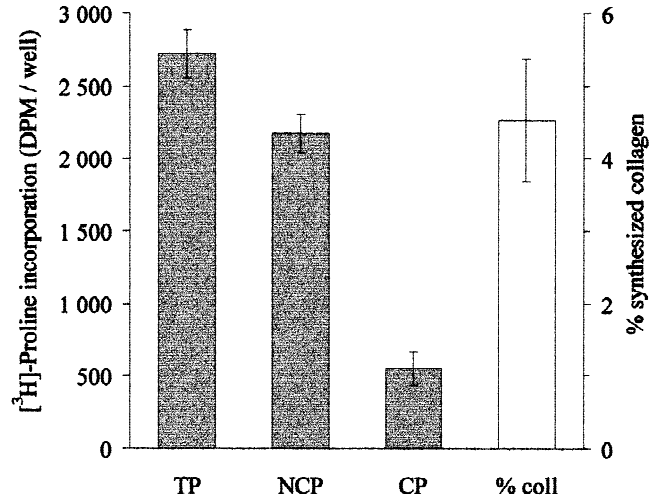

Figure 9. Collagen synthesis by cultured mantle cells. Cells were dissociated (procedure A, 0.1% pronase plus 0.1% collagenase), seeded at 4×10^6 cells per well in six-well culture plates, and cultured at 15°C in modified Hanks'-199 medium with 1.5 µl (1 µCi/µl) of L-[^{3}H]proline for 48 hours. Then, tritiated proline incorporation was determined by collagenase digestion as described in "Materials and Methods." The percentage of collagen (Coll) was calculated according to Peterkofsky and Diegelmann (1971). TP indicates total proteins; NCP, noncollagenous proteins; CP, collagen proteins; % coll, percentage of collagen. Each determination represents the average ± SD of triplicate culture assays, and a typical experiment was repeated at least three times.

proteins such as poly-L-lysines and fibronectin increased by 5-fold attachment of molluscan mantle cells. Similarly, Naganuma et al. (1994) reported hat poly-D-lysine and fibronectin enhanced attachment of dissociated cells from trochophore larvae of *Haliotis rufescens* by 5-fold. In contrast, Robledo and Cajaraville (1997) reported no significant effect of polylysine on enhancement of adhesive properties of mussel digestive gland cells. As pointed out, use of ECM molecules purified from invertebrates such as lectin from the ascidian (Odintsova et al., 1999) or mussel adhesive proteins could give better results (Deming, 1999).

In the present study, we have examined the viability of mantle cells during the first 6 days of culture by the MTT assay. Then, protein synthesis was also considered. Until almost 6 days of culture at 15°C, mantle cells showed a constant MTT response reflecting constant cellular metabolic activity. The de novo protein synthesis of cultured cells was measured by [^{3}H]leucine incorporation. For the same culture time, no significant variation in global protein synthesis was registered. This result confirms the growth potential of cells in vitro and shows that mantle cells can be used for the further studies presented here—namely, their capacity to synthesize in vitro PGs and collagen as components of the ECM.

For the first time, in this report, different PG/GAG

species, HS-PGs, CS/DS-PGs, and HA, were identified as being synthesized by molluscan cells in primary cultures. After 48 hours of labeling, [^{3}H]PG/GAG neosynthesis represented 43% of total [^{3}H]molecules. While HA seems to be present only in chordates (Nader et al., 1996), 6% of [^{3}H]glycosamine macromolecules synthesized were recovered as HA. In order to confirm the presence of HA in Mollusca, complementary investigations based on approaches such as immunocytochemistry should be performed. De novo synthesized alkali-sensitive [^{3}H]glycosamine macromolecules constituted 37%. A large part (82%) of these macromolecules are PGs with respect to their sensibility to HNO_2 or chondroitinase-ABC treatments. GAG material contained 45% heparan sulfate and 37% chondroitin/dermatan sulfate. The remaining 18% of β-eliminated [^{3}H]glycosamine macromolecules that have not been digested may correspond to others PGs, such as keratan sulfates, not investigated in this study or other *O*-glycosyl proteins. Early elution (0.1 M NaCl) on anion-exchange chromatography of these PGs indicated that most of them appeared as undersulfated molecules in comparison with homologous PGs purified from vertebrate tissues (0.4 M NaCl) (Thiébot et al., 1997). Such undersulfated molecules have already been described in invertebrates. In the mucus surrounding embryos of the gastropod mollusk, *Viviparus ater,* Volpi et al. (1998) partially purified and characterized a low-sulfated chondroitin sulfate PG. Accordingly, HS-PGs isolated from different molluscan tissues, especially from mantle, appeared to be undersulfated (Hovingh and Linker 1998). Thus, it seems that undersulfated GAG molecules are common in mollusks, and our results showing a synthesis of such undersulfated GAGs in cultured mantle cells agree with this notion.

The present study identified predominantly neosynthesized HS-PGs (45%) and CS/DS-PGs (37%) among the anionic [^{3}H]glycosamine-labeled material. These proteoglycans have been detected in all molluscan tissues analyzed, but the relative proportions of these two PGs varied from each tissue (Dietrich et al., 1983). Concerning the molluscan mantle, Hovingh and Linker (1998) showed that CS-PG was the major PG component (72 mg for 100-g dry weight mantle) in comparison with HS-PG (13 mg for 100-g dry weight mantle). Our cultured mantle cell model constitutes an in vitro system that differs from these studies. Thus, the discrepancy regarding the relative proportion of the two PGs registered in our study may correspond to the difference in experimental approaches—metabolic labeling from cultured cells rather than direct identification from tissue. Moreover, the amount of each category of PG depends on the conjunction of simultaneous rates of neosynthesis and catabolism occurring in cells. In addition, depending on the type and localization of PGs, the turnover may be quite variable. Thus, it is difficult to establish a direct comparison between values obtained in vitro and after a tissue extraction.

Second, it is well documented that changes in the expression of components of the ECM occur in relation to the adhesion of cells to their environment and their subsequent migration, which are regulated, in part, by their interaction with various components of the ECM. In fact, the initiation of cultures induces decreased or increased expression of ECM components, and modifications in the expression of components of the ECM have been observed in relation to the time and conditions of culture. PGs, especially HS-PGs, are molecules that promote cell adhesion and migration. HS-PGs are necessary for the formation of stable adhesion sites on substrates (Har-El and Tanzer, 1993; Wight et al., 1992). In invertebrates a role for HS-PGs in axon guidance has been found in cell cultures and in embryos of the cockroach *Periplaneta americana* (Carbonetto et al., 1983). In our study, after the initiation of cultures, part of the mantle cells adhered on the flask or formed clusters. In addition, some cells migrated and flattened on the surface of flasks. So, it is not surprising that during the first hours of culture we registered a relatively abundant synthesis of HS-PGs. In a recent report, Odintsova et al. (1999) have shown that ascidian lectin promotes both adhesion and growth of cultivated marine cells. Future investigations using an in vitro model such as the one described in this study should help elucidate the relation between molluscan cell adhesion or growth and synthesis of PG components.

In this study, we examined and quantified collagen synthesis by cultured mantle cells. During the first 48 hours, de novo synthesis represents 4.52% of the total protein synthesis. In invertebrates, an in vitro study focusing on collagen synthesis has been conducted in sea urchin micromeres (Blankenship and Benson, 1984; Benson et al., 1990; Wessel et al., 1991). On days 1–5 of culture, collagen synthesis ranged from 0.5% to 5% of the total protein synthesis (Benson et al., 1990). Thus, the de novo synthesis observed in cultured mantle cells is comparable to that reported for sea urchin micromeres. Nevertheless, as mantle is composed of different cell types, the value registered in our study reflected a mean value. One could hypothesize that the rate of collagen synthesis is heterogeneous among the different types of cells, with the synthesis capacity varying

with cell type. The question arising concerns the identification of the cells producing collagen and/or PGs. To date, no precise answer is available; however, further studies based on the cultivation of cells enriched in one or another type may provide some information. By Northern blot analysis, collagen-encoding RNAs have been identified in the mantle of the abalone *Haliotis discus* (Yoneda et al., 1999). Our results agree with the synthesis of collagen molecules by mantle cells of mollusks as ECM components.

Among their multiple functions, collagen and PGs act as tissue organizers, influencing cell growth and migration (Hardingham and Fosang, 1992; Wight et al., 1992; Har-El and Tanzer, 1993; Taipale and Keski-Oja, 1997). Concerning the maturation of specialized tissues and more precisely the process of mineralization, Sud et al. (1998) have pointed out that the use of cell cultures from the mantle of abalone presents a new approach for further in vitro studies in molluscan biomineralization. Our present demonstration indicates that mantle cells have the ability to produce in vitro PGs/GAGs and collagen as ECM components and agrees with this notion. Such a matrix synthesis is considered as the first event of molluscan shell elaboration. Proteoglycans have been implicated as fundamental components of mineralized tissues in both vertebrates (Boskey, 1996) and invertebrates, because PGs have been identified in the sea urchin shell (Manouras et al., 1991) and in the organic shell matrix of the snail *Biomphalaria glabrata* (Marxen et al., 1998). Moreover, it seems that at least one of the nacre-insoluble matrix proteins from abalone shell is collagenase-sensitive (Schäffer et al., 1997). Thus, collagen may represent another key component of molluscan biomineralization.

Both PGs/GAGs and collagen participate in molecular events that regulate cell adhesion, migration, and proliferation (Hardinghan and Fosang, 1992; Wight et al., 1992; Har-El and Tanzer, 1993; Taipale and Keski-Oja, 1997). The ability of the extracellular matrix to bind growth factors such as transforming growth factor β (TGF-β) or fibroblast growth factor (FGF) (Taipale and Keski-Oja, 1997) is a major mechanism regulating growth factor activities and subsequent cellular events. In turn, growth factors can modulate the ECM synthesis. In vertebrates, in vitro systems have provided the opportunity to examine effects of growth factors such as insulin-like growth factor (IGF) or TGF-β on the synthesis of ECM components (Galéra et al., 1992; Thiébot et al., 1997). First, TGF-β (Franchini et al., 1996; Kletsas et al., 1998) and insulin-like peptides (ILPs) have been detected in mollusks (Thorpe and Duve, 1984; Ebbenrink et al., 1989; Kellner-Cousin et al., 1994). Second, insulin or members of the family of insulin-like peptides stimulate both body and shell growth as well as protein synthesis in hemocytes and in mantle cells from *Haliotis tuberculata* (Sevala et al., 1993; Lebel et al., 1996; Giard et al., 1998). So, mantle cell cultures may be an excellent system to analyze the effect of growth factors on the synthesis of PGs/GAGs and collagen in mollusks.

In conclusion, expression and regulation of collagen and PGs play important roles in cell-cell and cell-ECM interactions in marine invertebrates (Brandan et al., 1992; Volpi et al., 1998). Future studies focusing on in vitro models should provide knowledge about new aspects of molluscan cell signaling, in relation to ECM components.

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