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SHORT COMMUNICATION

Spatiotemporal distribution patterns of oligosaccharides during early embryogenesis in the starfish *Patiria pectinifera*

Takuya Doihara · Yuji Miguchi · Kyojy Miyawaki · Tetsuya Shimokawa · Fumihiko Hamada · Naoto Kobayashi · Seiji Matsuda

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Abstract To examine embryogenic mechanisms in the starfish *Patiria* (*Asterina*) *pectinifera*, we histochemically analyzed several larval stages using Alcian Blue (AB, which stains acidic mucins), Periodic Acid Schiff (PAS, which stains neutral mucins), and 21 types of lectins. Carbohydrate distribution patterns were observed in the cytoplasm, basement membrane, and blastocoel as follows: (1) The first group of lectins showed granular signals in the mesendodermal cells, and these lectins may be useful as mesendoderm markers. (2) The second class of lectins showed diffuse signals across the entire cytoplasm from the hatched blastula until the mid gastrula. These signals became localized to the basal cytoplasm of archenteron cells at the early bipinnaria. (3) Lectin reactivity in the basement membrane peaked at the early-to-mid gastrula and was nearly gone by the early bipinnaria. These results suggest the existence of various substances in the basement membrane and imply the importance of these substances during archenteron elongation and the induction of mesenchyme differentiation. (4) Signal colors with AB–PAS double staining in the blastocoel changed from magenta (by PAS staining) into blue (by AB staining) during these stages, thus, indicating that mucin located in the blastocoel changed from neutral to acidic. The most significant part of this report is the first description regarding temporal changes in the characteristics of intra- and extracellular components with the combination of many different lectins and stains.

Keywords Starfish · Embryogenesis · Lectin · Mucin · Histochemistry

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T. Doihara · Y. Miguchi · T. Shimokawa · F. Hamada · S. Matsuda
Department of Anatomy and Embryology,
Ehime University Graduate School of Medicine,
Tohon, Ehime, Japan

K. Miyawaki (✉)
Shikoku-chuoh Medical & Welfare General Collage,
Shikoku-chuoh, Ehime, Japan
e-mail: gastrula@rwf.ac.jp

N. Kobayashi
Medical Education Center, Ehime University School of Medicine,
Tohon, Ehime, Japan

Introduction

Echinoids, which produce large numbers of eggs and have clear larval bodies, have been used in embryological research for a long time. Many reports about germ layer-specific molecular markers have been published (e.g., Levine and Davidson 2005). In contrast, asteroids, which share the same characteristics as echinoids, were not used until the 1970s because of difficulties in obtaining mature eggs. Thus, there are few reports regarding germ layer-specific markers in asteroids. The starfish *Patiria* (*Asterina*) *pectinifera* has numerous characteristics similar to sea urchins, making it favorable for study. For example, this starfish has gametes that can be obtained for an entire year, fertilized eggs that cleave equally, and clear cytoplasm that allows for direct observation of the larvae through the developmental stages (Kuraishi and Osanai 1994).

Various molecules synthesized in the cells are decorated with sugar chains, and such modifications play various roles in cell–cell interactions. Specifically, electric cell surface charges produced by the carbohydrate chains are involved in cell adhesion (Hakomori 2000). Furthermore, carbohydrate

chains play important roles in cell recognition and cell differentiation during embryogenesis (Muramatsu 1988).

Lectins are frequently used to analyze sugar chain distribution patterns in many animal tissues (Contini et al. 1992; Jiang et al. 2004). Lectins are classified by the binding specificity with the monosaccharides. However, lectins showing the same specificity to monosaccharides may have different affinities to larger saccharides and derivative substances (Dam et al. 2000). For example, *Bauhinia purpurea* agglutinin, which recognizes *N*-acetylgaractos-amine (GalNAc), stains the entire renal tubule, but *Dolichos biflorus* agglutinin (DBA), which also recognizes GalNAc, stains the collecting duct during histochemical staining of the mammalian kidney. Thus, given their variation and sensitivity of the stainability, lectins are a superior tool for evaluating stain specimens. Furthermore, Concanavalin A (Con A) treatment causes sea urchin embryos to dissociate into single cells, and the reaggregation of those cells is inhibited (Lallier 1972). Blastula formation in starfish embryos is also inhibited by Con A (Kyoizumi and Kominami 1980). These reports indicate that carbohydrate chains play important roles during embryogenesis. Thus, we analyzed the distribution pattern of carbohydrate chains during early embryogenesis in *P. pectinifera*, using 21 kinds of lectins, Alcian Blue (AB), and Periodic-Acid Schiff (PAS) staining to obtain fundamental information about the relationship between carbohydrate chain expression patterns and morphogenesis in early embryogenesis.

Materials and methods

Animals and gametes

Adult *P. pectinifera* were collected at several points along the coast of Japan (Aomori, Kanagawa, Okayama, and Hiroshima Prefectures) during the breeding season. Starfish were kept in an aquarium supplied with cold seawater (10±1°C).

Oocyte maturation and artificial insemination were performed following the methods of Miyawaki et al. (2003). Embryos were cultured at 20°C in artificial seawater.

Preparation of specimens

Hatched blastulae, early-to-mid gastrula, mid gastrula, and early bipinnaria were used in this study.

Embryos were fixed by rinsing three times with ice-cold 100% methanol and twice with ice-cold 100% ethanol. Fixed specimens were incubated with polyester wax containing 50% ethanol at 40°C for 1 h. The specimens were then completely embedded in polyester wax by repeatedly removing the supernatant, adding 100% polyester wax, and incubating at 40°C for 1 h. Embedded embryos were cut into 5-μm sections using a microtome and were examined histochemically.

Mucin histochemistry

First, we performed AB staining (stains for acidic mucins) and PAS staining (stains for neutral mucins) to obtain the basic spatiotemporal carbohydrate patterns. Dewaxed slides were rinsed with 3% acetic acid for 3 min and treated with AB 8GX (pH 2.5) for 1 h. After rinsing twice with 3% acetic acid, slides were washed in running tap water for 3 min and counterstained with Kernechtrot.

A PAS reaction was performed, and slides were treated with Schiff's reagent for 15 min followed by 0.5% periodic acid treatment for 5 min. Slides were then washed with distilled water. After treating the slides three times with a $NaHSO_3$ solution for 3 min, the slides were washed in running tap water for 3 min and counterstained with hematoxylin. AB–PAS double staining was performed, so that the PAS reaction continuously occurred after AB staining.

Lectin histochemistry

To detect distribution patterns of the carbohydrate chains, specimens were histochemically stained using 21 kinds of biotinylated lectins (lectin screening kit, Vector Laboratories, Burlingame, CA, USA), a VECTASTAIN ABC standard kit (Vector), and 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich, St. Louis, MO, USA). Lectins and their respective binding specificities and applied concentrations are listed in Table 1. We divided the lectins into three classes according to their reactivities to cytoplasm (Table 2). Staining was performed following the methods previously described by Saito and Taniguchi (2000). Nuclei were counterstained with methyl green.

Absorptive experiment

We performed an absorptive experiment to check lectin specificity. Before staining, each lectin was incubated with 100× reducing sugar (fucose, galactose, *N*-acetylgalactosamine, *N*-acetylglucos-amine, mannose, *N*-acetylneuraminic acid) for 1 h at room temperature and used for lectin histochemistry. In this absorptive experiment, no positive signal was detected (data not shown).

Results and discussion

Mucin histochemistry

No noticeable AB reactions were seen in the hatched blastula (Fig. 1a, a′). However, reactions were observed in

Table 1 Lectins used in this study

Lectin	Binding specificity	Concentration (μg/ml)
Wheat germ agglutinin (WGA)	β-GlcNAc>α-NeuNAc	1.0
Succinylated wheat germ agglutinin (s-WGA)	β-GlcNAc	2.5
Lycopersicon escuientum lectin (LEL)	β-GlcNAc	0.5
Solanum tuberosum lectin (STL)	β-GlcNAc	2.0
Datura stramonium lectin (DSL)	β-GlcNAc	0.29
Bandeiraea simplicifolia lectin-II (BSL-II)	α, β-GlcNAc	6.7
Dolichos biflorus agglutinin (DBA)	α-GalNAc	50
Soybean agglutinin (SBA)	α,β-GalNAc, Gal	1.0
Bandeiraea simplicifolia lectin-I (BSL-I)	α-GalNAc, α-Gal	10
Vicia villosa agglutinin (VVA)	α,β-GalNAc	2.0
Sophora japonica agglutinin (SJA)	β-GalNAc, β-Gal	67
Ricinus communis agglutinin-I (RCA-I)	β-GalNAc, β-Ga1	2.0
Jacalin	Ga1actosyl-β-GalNAc	0.067
Peanut agglutinin (PNA)	Galactosyl-β-GalNAc	10
Erythrina cristagalli lectin (ECL)	Ga1actosyl-β-GlcNAc	5.0
Ulex europaeus agglutinin-I (UEA-I)	α-Fuc	20
Concanavalin A (ConA)	α-Man	0.13
Pisum satibum agglutinin (PSA)	α-Man	2.0
Lens culinaris agglutinin (LCA)	α-Man	1.4
Phaseolus vulgaris agglutinin-E (PHA-E)	Oligosaceharide	1.0
Phaseolu vulgaris agglutinin-L (PHA-L)	Oligosaccharide	1.0

Fuc fucose, *Gal* galactose, *GaINAc N*-acetylgalactosamine, *GIcNAc N*-acetylglucosamine, *Man* mannose, *NeuAc N*-acetylneuraminic acid

Table 2 Lectin binding pattern of starfish larvae

Group	Lectins	Hatched blastura				Early-to-mid gastrula				Mid gastrula				Early bipinnarias			
		A-CP	CP	BM	BC	A-CP	CP	BM	BC	A-CP	CP	BM	BC	A-CP	CP	BM	BC
1	SBA	++	−	−	−	++	±	−	−	++	±	−	−	++	−	−	−
1	PNA	++	−	−	−	++	±	±	−	+	±	−	−	+	±	−	−
1	BSL-I	++	+	−	±	++	++	+	±	++	+	+	+	++	±	−	−
1	RCA-I	++	+	−	±	++	+	+	+	++	+	±	+	+	±	−	−
1	VVA	++	++	−	±	++	+	−	±	++	+	−	±	++	+	−	±
1	DBA	+	++	−	+	+	++	±	+	+	+	−	±	+	+	−	±
1	Jacalin	+	+	−	−	++	+	++	±	+	+	±	+	++	±	+	++
2	ConA	−	+++	−	++	−	+++	+	++	−	+++	−	+	−	+++	−	++
2	BSL-II	−	+++	+	++	−	+++	+	++	−	+++	−	±	−	+++	−	++
2	STL	−	+++	+	++	−	++	+	+	−	+++	+	±	−	+++	−	+
2	LCA	−	+++	±	++	−	++	++	+	−	+++	+	±	−	++	−	−
2	LEL	−	+++	+	++	−	++	±	+	−	++	−	±	−	+++	−	+
2	PSA	−	+++	±	+	−	++	−	+	−	++	+	+	−	++	−	±
3	WGA	−	+	−	−	−	±	++	−	−	+	+	−	−	+	−	−
3	SJA	−	+	±	−	−	+	++	−	−	+	+	+	−	±	+	±
3	ECL	−	+	−	±	−	+	++	−	−	+	±	−	−	±	−	−
3	PHA-E	−	+	−	−	−	+	++	−	−	+	+	+	−	+	−	−
3	PHA-L	−	+	−	−	−	+	+	−	−	±	±	−	−	+	−	−
3	s-WGA	−	+	−	−	−	+	±	−	−	+	±	−	−	+	−	±
3	DSL	−	+	−	−	−	±	−	−	−	+	−	±	−	±	−	−
3	UEA-I	−	++	−	−	−	+	+	−	−	+	−	±	−	±	−	−

A-CP apical area of cytoplasm, *CP* cytoplasm, *BM* basement membrane, *BC* blastocoel, − negative staining, ± weak staining, + moderate staining, ++ strong staining, +++ very strong staining

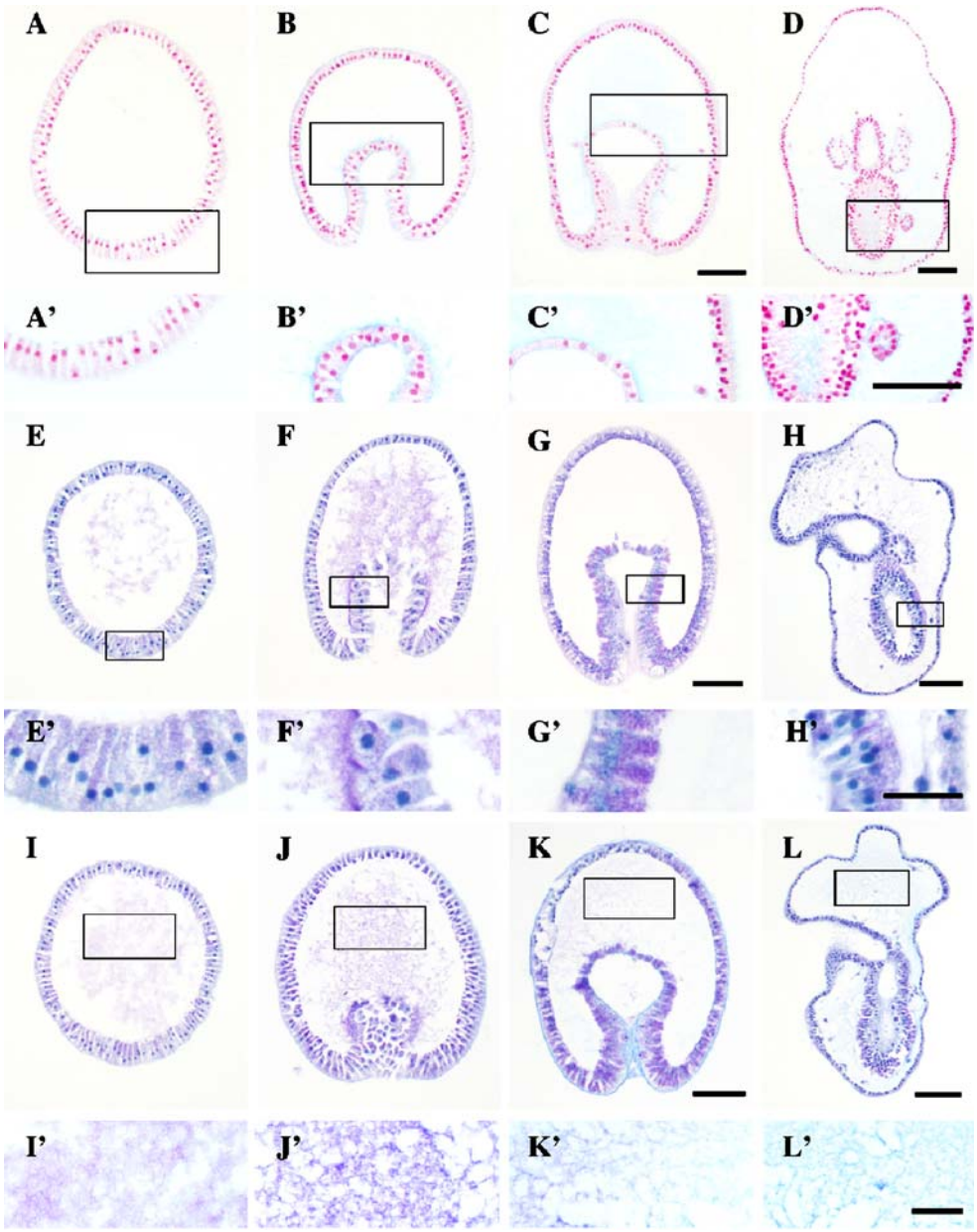

Fig. 1 Mucin staining of starfish larvae. **a**–**d** AB staining. **e**–**h** PAS staining. **i**–**l** AB–PAS double staining. **a**, **e**, **i** Hatched blastula. **b**, **f**, **j** Early-to-mid gastrula. **c**, **g**, **k** Mid gastrula. **d**, **h**, **l** Early bipinnaria. **a′**–**l′** Higher magnification of the areas enclosed by *rectangles* in (**a**–**l**). AB reaction was observed in the basement membrane and the hyaline layer of the archenteron (**b**, **b′**, **c**, **c′**), blastocoel (**c**, **c′**, **d**, **d′**), and inside the digestive organ (**d**, **d′**). Granular PAS reactions were seen in the cytoplasm (**e**–**h**, **e′**–**h′**). PAS reactions were also seen in the basement membrane of the archenteron during the early-to-mid gastrula (**f**, **f′**) and in the blastocoel (**e**–**h**). Localization of granular reactions in the cytoplasm on the basal side was seen during the early bipinnaria (**h**, **h′**). During hatched blastula and early-to-mid gastrula, PAS-positive magenta reactions were observed in the blastocoel (**i**, **j**, **i′**, **j′**). In the mid gastrula, purple reactions merged with AB-positive blue reactions, and PAS-positive magenta reactions were observed in the blastocoel (**k**, **k′**). In the early bipinnaria, AB-positive blue reactions were observed in the blastocoel (**l**, **l′**). Nuclei were counterstained with Kernechtrot (**a**–**d**) and hematoxylin (**e**–**l**). *Bar* 50 μm (**a**–**l**, **a′**–**d′**), 20 μm (**e′**–**l′**)

the basement membrane and hyaline layer of the archenteron during early-to-mid gastrula (Fig. 1b, b′), mid gastrula (Fig. 1c, c′), and in the blastocoel at the mid gastrula (Fig. 1c, c′). Reactions observed in the blastocoel around the archenteron tip were particularly remarkable. In the early bipinnaria, AB signals were observed in the basement membrane of the archenteron, blastocoel, and inside of the digestive organ (Fig. 1d).

PAS-positive granular reactions were seen in the cytoplasm during all stages (Fig. 1e–h, e′–h′). Reactions were also seen in the blastocoel at the hatched blastula (Fig. 1e). In the early-to-mid gastrula, PAS-positive reactions were observed in the basement membrane of archenteron cells (Fig. 1f, f′). Strong reactions were also observed in the blastocoel (Fig. 1f). At the early bipinnaria, PAS-positive reactions were also seen in the blastocoel (Fig. 1h). PAS-positive granular reactions were localized to the basal side of the cytoplasm in archenteron cells (Fig. 1h′).

In the AB–PAS double-stained slides, PAS-positive magenta reactions recognizing fibrous structures were observed in the blastocoel during the hatched blastula and early-to-mid gastrula (Fig. 1i, i′, j, j′). At the mid-gastrula stage, purple fibrous reactions merged with AB-positive blue reactions, and PAS-positive magenta reactions were observed in the blastocoel (Fig. 1k, k′). In the early bipinnaria, AB-positive blue reactions recognized fibrous structures similar to previous stages in the blastocoel (Fig. 1l, l′). These results suggest that mucin-containing fibrous structures in the blastocoel gradually changed from neutral to acidic during the archenteron formation.

Lectin histochemistry in the cytoplasm

Reactivities in the cytoplasm were divided into three groups as follows (Table 2). Figure 2 shows typical staining pattern for each of the three groups. First, granular signals were observed in the apical cytoplasm of vegetal plate cells (Fig. 2a), archenteron cells (Fig. 2b–d), coelomic pouch cells, and mesenchyme cells (Fig. 2d). These signals might be recognized Golgi apparatuses, given that Terasaki (2000) reported that such organelles accumulated at the apical cytoplasm after the hatched blastula stage. Since the lectins (SBA, PNA, BSL-I, RCA-I, VVA, DBA, and Jacalin) showing the above staining pattern have binding specificity to GalNAc (Table 1), it is thought that reactive products have GalNAc in their carbohydrate chains. Strong or moderate granular signals with the first group of lectins were observed during all stages. These granular signals were only observed on the apical side by SBA- and PNA-staining; however, the other lectins in this group showed moderate or weak blur-like signals in the cytoplasm during all stages (Table 2). SBA has been reported to be a marker of the mesendoderm in starfish during early embryogenesis (Shimizu et al. 1995). Our results show that PNA, BSL-I, RCA-I, VVA, DBA, and Jacalin stain the mesendoderm during all stages. Thus, these lectins may be useful as mesendoderm markers.

In the sea urchin embryo, synthesis levels of N-linked glycoproteins are low during the early cleavage stage and rise in the late blastula stage (Heifetz and Lennarz 1979). Additionally, treatment with tunicamycin, an inhibitor of N-linked glycosidation, blocked gastrulation in sea urchin

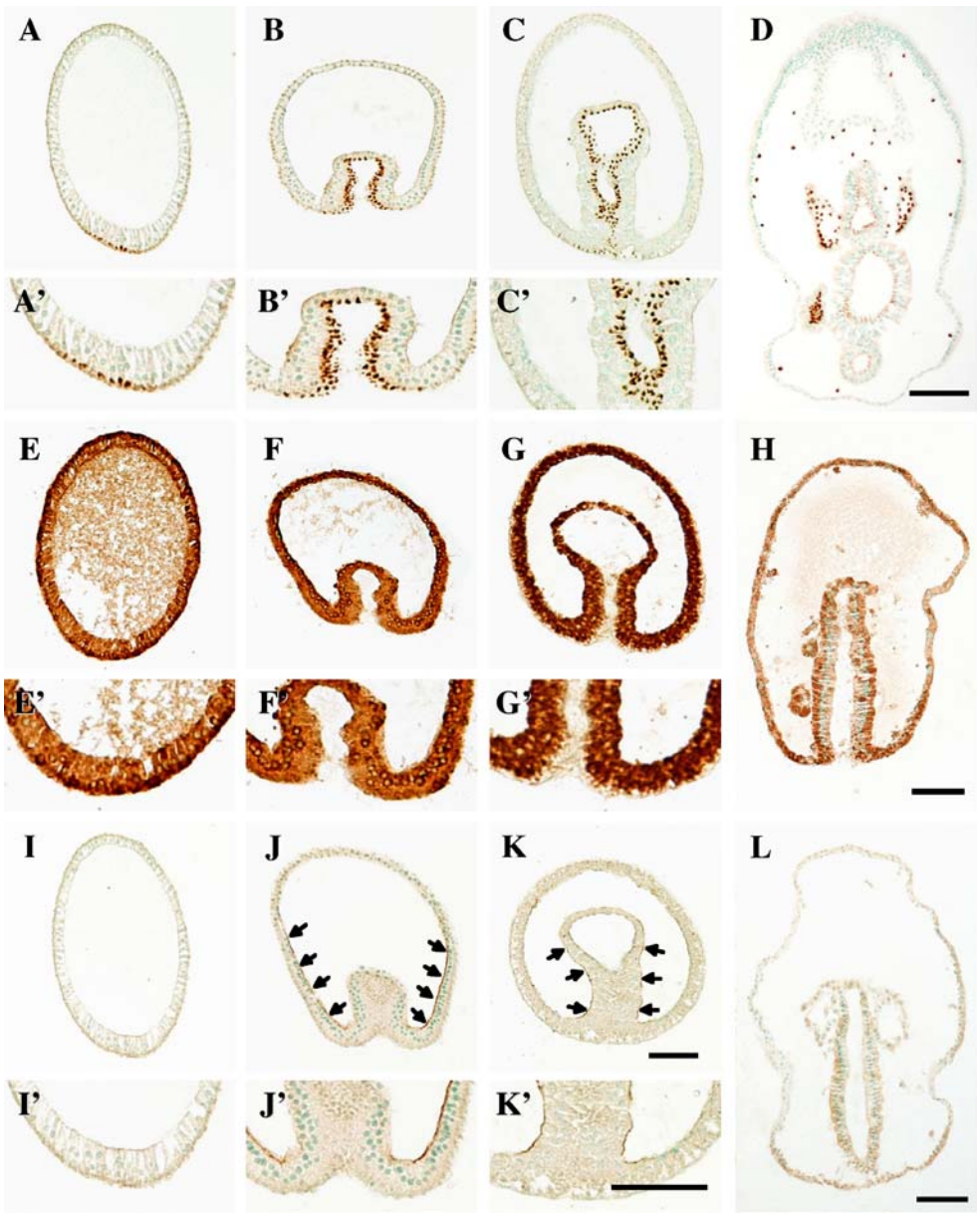

Fig. 2 Lectin staining of starfish larvae. **a**–**d** SBA lectin-stained larvae. **e**–**h** Con A lectin-stained larvae. **i**–**l** WGA lectin-stained larvae. **a**, **e**, **i** A hatched blastula. **b**, **f**, **j** An early-to-mid gastrula. **c**, **g**, **k** A mid gastrula. **a′**–**c′**, **e′**–**g′**, **i′**–**k′** Higher magnification of (**a**–**c**, **e**–**g**, **i**–**k**). **d**, **h**, **l** An early bipinnaria. Positive granules were observed in the apical side of the vegetal plate (**a**, **a′**) archenteron cells (**b**, **b′**, **c**, **c′**, **d**), coelomic pouch cells, and mesenchyme cells (**d**). The entire cytoplasm was very strongly labeled with Con A lectin staining (**e**–**h**). The entire cytoplasm was moderately or weakly labeled with WGA lectin staining (**i**–**l**). Recognition sites were observed in some areas of the basement membrane at several stages (**j**, **k**, *arrow*). The area overlying the invaginating archenteron was strongly labeled (**j**, *arrow*). Nuclei were counterstained with methyl green. **a**–**c**, **e**–**g**, **i**–**k** and **a′**–**c′**, **e′**–**g′**, **i′**–**k′** are the same magnification. *Bar* 50 μm

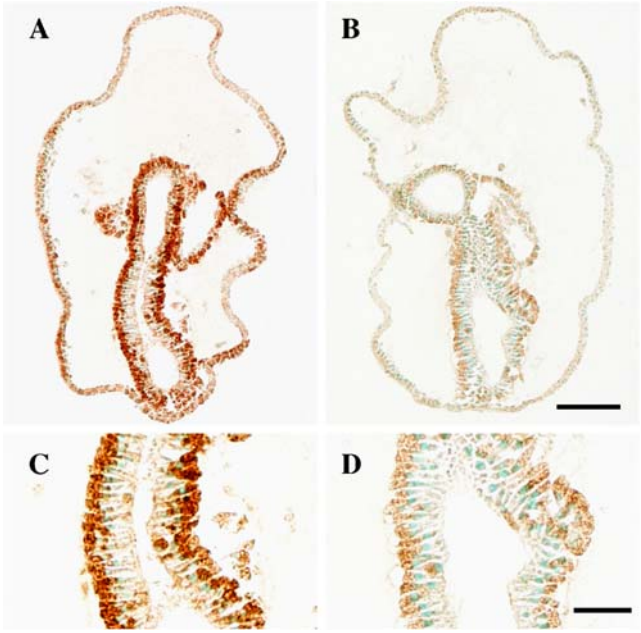

Fig. 3 Lectin staining pattern of early bipinnaria. **a** PSA lectin-stained larva. **b** PHA-L lectin-stained larva. **c**, **d** Higher magnification of (**a**, **b**). Recognition sites in the cytoplasm of the archenteron cells were distributed on the basal side (**c**, **d**). Nuclei were counterstained with methyl green. **a**, **b** and **c**, **d** are the same magnification. *Bar* 50 μm (**a**, **b**), 20 μm (**c**, **d**)

embryogenesis (Heifetz and Lennarz 1979). The synthesis of SBA-positive glycoproteins in the vegetal plate later disappeared, and gastrulation was blocked by albuside B treatment in the starfish embryo (Shimizu et al. 1995). From these reports and our observations, we suggest that N-linked glycoproteins, including those binding to first-group lectin, play important roles in gastrulation.

The second group of lectins (Table 2: Con A, BSL-II, STL, LCA, LEL, and PSA) showed strong labeling throughout the entire cytoplasm as indicated in Fig. 2e–h (stained with Con A). As shown in Fig. 3, all second-group lectins and some third-group lectins (PHA-E, PHA-L, s-WGA, DSL, and UEA-I) recognized the basal cytoplasms of the early bipinnaria archenteron cells. These results suggest that the glycoconjugated substances were dispersed to the cytoplasm accumulated to the basal side of the cytoplasm of archenteron cells in the early bipinnaria stage. These lectins may also recognize neutral mucins because these lectins stained the same regions as PAS staining during all developmental stages.

The third group of lectins (Table 2: WGA, SJA, ECL, PHA-E, PHA-L, s-WGA, DSL, and UEA-I) exhibited moderate or weak staining of the cytoplasm through all stages (Fig. 2i–l).

Lectin histochemistry in the basement membrane

As shown in Table 2, classifications according to reactivities in the cytoplasm do not completely accord with the staining patterns of the basement membrane, but some rules do exist. In general, signals in the basement membrane were the most intense during the archenteron elongation stage and gradually decreased and finally disappeared at the early bipinnaria stage (except Jacalin and SJA; Table 2). At the hatched blastula stage, the basement membrane was labeled moderately or weakly with the second group of

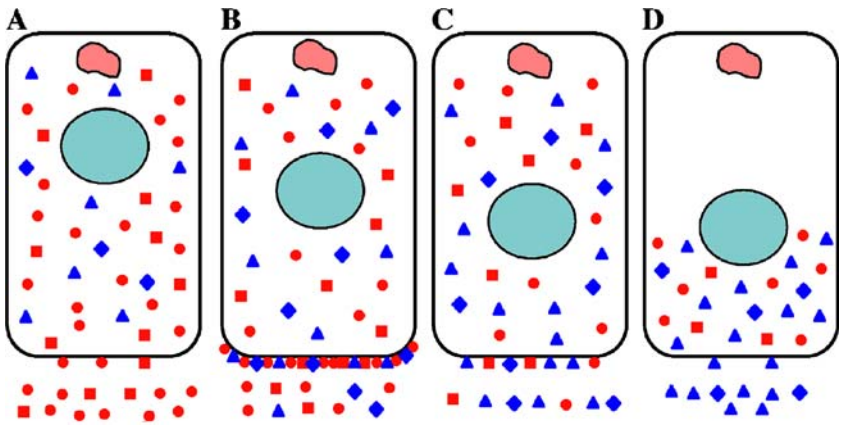

Fig. 4 Schematic representation of carbohydrate chain distribution patterns in archenteron cells during early embryogenesis in the starfish. The large irregular object shows granular reactions seen with the first-group lectins. Other reactions with lectins are shown by smaller symbols (*circle*, *triangle*, *square*, *diamond*). *Upper and lower parts of the cell* show the apical and basal sides, respectively. Differences in *symbol colors* indicate the characters of the intra- and extracellular matrix (*red* neutral, *blue* acidic), and differences in *symbol shapes* indicate kinds of carbohydrate chains (not specified). *Numbers of symbols* correspond to the number of plus signs in Table 2. **a** Hatched blastula. **b** Early-to-mid gastrula. **c** Mid gastrula. **d** Early bipinnaria. Many kinds of carbohydrate chains are produced in the cytoplasm during the hatched blastula to mid-gastrula stage (**a**, **b**, **c**). These substances are located on the basement membrane during the early-to-mid gastrula to the mid gastrula (**b**, **c**). Substances produced in the cytoplasm are limited to the basal side and substances in the blastocoel are limited to acidic mucins (**d**). The substances distributed in the blastocoel gradually change from the hatched blastula to the early bipinnaria (**a**–**d**)

lectins, excluding Con A. The basement membrane was not labeled with any first- and third-group lectins (except SJA; Table 2). In the early-to-mid gastrula, signals were seen with all lectins except SBA, VVA, PSA, and DSL. In particular, Jacalin, LCA, WGA (Fig. 2j, j′), SJA, ECL, and PHA-E were strongly labeled within the basement membrane (Table 2). Strong signals in the area overlying the invaginating archenteron were observed with WGA (Fig. 2j, j′), RCA-I, and UEA-I staining (data not shown).

Signals with AB and PAS were also observed in the basement membrane during the early-to-mid-gastrula stage (Fig. 1b, b′, f, f′). These results show that many kinds of mucins are localized in the basement membrane during the early-to-mid-gastrula stage.

As shown in Fig. 2j and j′, some lectins reacted with the basal lamina around the elongating archenteron. Ingersoll and Ettensohn (1994) reported that ECM-1, a monoclonal antibody against the N-linked glycoprotein in the basal lamina, reacted with the basal lamina around the invaginating archenteron in sea urchin embryos. Given these results, we speculate that materials accumulating around the archenteron are necessary to elongate the archenteron.

Lectin histochemistry in the blastocoel

At the hatched blastula stage, the fibrous structures in blastocoel were stained strongly with the second-group lectins (except PSA) but not the third group (except ECL; Fig. 2e; Table 2). Some first-group lectins were also stained moderately or weakly (Table 2). In the early-to-mid gastrula, almost all first- and second-group lectins (except SBA and PNA) stained netlike structures in the blastocoel. In particular, the mesh-like structures were strongly recognized by ConA (Fig. 2f) and BSL-II (Table 2). At the mid gastrula, some third-group lectins also stained moderately or weakly (Table 2). In the early bipinnaria, the positive signals in blastocoel intensified with Jacalin-, ConA- (Fig. 2h), and BSL-II-staining compared to the previous stage. Conversely, the intensity of VVA, DBA, STL, LEL, PSA, SJA, and s-WGA staining decreased (Table 2).

Further, Jacalin-staining signals in the blastocoel increased gradually from the hatched blastula to the early bipinnaria (Table 2). In contrast, LCA-staining showed that reactions decreased gradually from the hatched blastula to the early bipinnaria (Table 2). These results suggest that Jacalin reacts with galactosyl-β-GalNAc-rich acidic mucin, and LCA reacts with galactosyl-β-GlcNAc-rich neutral mucin.

The ECM provides cells with an environment to shapes the epithelia (Kaneko et al. 2005) and also acts as a substrate for cell adhesion and guidance (McClay and Ettensohon 1987) in echinoderm larvae. In *Pisaster ochraceus* larvae, Maghsoodi and Crawford (2005) suggested that the PM-2 epitope which is a content of acidic mucin of blastocoelic ECM containing N-linked carbohydrate chains was necessary for proper formation of the blastocoel, movement of mesenchyme cells, and so on. Our study was consistent with these reports in many respects. Increased reactivity to the acidic mucins in the blastocoel from the early-to-mid gastrula to the early bipinnaria may suggest the importance of acidic mucins in the blastocoel during migration and guidance of the mesenchyme cells, similar to the above reports.

In conclusion, our results suggest that many kinds of substances are produced in the cytoplasm during the hatched blastula to mid-gastrula stages (Fig. 4a–c). These substances that are composed of many kinds of carbohydrate chains, primarily acidic mucins, which were apparently located on the basement membrane from the early-to-mid gastrula to the mid-gastrula stages (Fig. 4b, c). Finally, substances produced in the cytoplasm accumulated on the basal side, and materials distributed to the blastocoel were limited to acidic mucins (Fig. 4d).

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