CELL STRUCTURE AND FUNCTION 22: 413–420 (1997)

Binding of Lectins to Novel Migration Promoters on Cardiac Mesenchymal Cells in the Chick

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Key words: Glycoprotein/extracellular matrix/development/heart

ABSTRACT. **Chicken serum promotes migration of cardiac mesenchymal cells of chick embryos *in vitro*. In the present study, migration promotion of unknown migration promoters in chicken serum was examined by using lectins. Cardiac mesenchymal cells of the conotruncal and atrioventricular cushions were cultured on collagen type-I gel with medium including chicken serum. A concentration (100 μg/ml) of Concanavalin A (Con A), peanut agglutinin (PNA), pisum sativum agglutinin (PSA), soybean agglutinin (SBA) or wheat germ agglutinin (WGA) was added to the medium. Con A, PSA, and WGA inhibited migration, while PNA and SBA did not affect migration of cardiac mesenchymal cells. WGA inhibited migration in a concentration dependent manner. Preincubation of WGA with specific binding monosaccharides (α-D-N-acetylglucosamine and α-D-N-acetylneuraminic acid) clearly reduced the inhibition ability of WGA, while preincubation of Con A and PSA with α-D-mannose and α-D-glucose did not. On the other hand, Con A-binding proteins, eluted from a Con A affinity column with the buffer including α-D-mannose and α-D-glucose, promoted migration of cardiac mesenchymal cells, as did WGA-binding proteins. These proteins promoted migration in a concentration dependent manner. Western blotting showed that PSA bound the subunits of collagen type-I, but ConA and WGA did not. In migration inhibition assays by monosaccharides, only N-acetylneuraminic acid inhibited migration of cardiac mesenchymal cells. These results suggested that chicken serum contains novel migration promoters for chick cardiac mesenchymal cells. The promoters are proposed to have the terminal N-acetylglucosamine, N-acetylneuraminic acid, and glucose and/or mannose residues.**

Early in the development of birds and mammals, the heart is a single muscular tube. Primordia of intracardiac structures, the endocardial cushions, develop at the next stage of development. The conotruncal (CT) and atrioventricular (AV) cushions are major endocardial cushions contributing to conotruncal and atrioventricular septation (36). These two cushions consist of rich extracellular matrices and mesenchymal cells. Mesenchymal cells of the CT and AV cushions are derived from the endocardium, and these endocardium-derived cardiac mesenchymal (CM) cells migrate in the cushion tissue toward the myocardium (15, 25, 31, 32). Although CM cell migration would concern normal cardiogenesis, essential understanding of the mechanisms of such migration has been limited.

Recent studies have clarified cell migration involving glycoproteins such as fibronectin (34, 42), laminin (10, 27), collagen (10), vitronectin (43) and tenascin (8, 17, 22, 40). The interaction between cells and these glycoproteins is usually mediated by certain amino-sequence of a peptide region, such as the RGD (Arg-Gly-Asp) sequence in fibronectin, vitronectin and tenascin, or the YIGSR (Tyr-Ile-Gly-Ser-Arg) sequence in laminin (46). Precise mechanisms by which such peptide sequences mediate migration remain obscure. Certainly exogeneous fibronectin promotes CM cell migration, and CM cells themselves express a fibronectin gene during their migration period (14). The RGD peptide, however, does not disturb CM cell migration *in vitro* (10). Similarly, the YIGSR peptide perturbs migration of CM cells on laminin, but it does not affect migration of CM cells when cells are cultured on a mixture substrate of laminin and collagen type-I (10). The anti-fibronectin antibody perturbs migration of CM cells when cells are cultured on fibronectin alone (43), but it does not do so when cells are cultured with serum (44). On the other hand, whereas the anti-fibronectin antibody inhibits mi-

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gration of CM cells *in situ* by microinjection into the endocardial cushions, the RGD peptide cannot completely inhibit migration (26). These findings suggest that fibronectin, laminin and collagen type-I certainly play an important role for CM cell migration, but CM cells and these matrices do not interact in a simple manner. Involvement of these molecules in CM cell migration are probably partial and other factors may certainly function as well.

Glycoconjugates are possibly involved in controlling cell motility. The terminal fucose (Fuc) residue linking to galactose (Gal) of cell surface molecules has been suggested to play an important role in cell migration (33). Terminal sugar residues of extracellular matrices may also mediate migration of CM cells. It has been reported that cell surface galactosyltransferase (GalTase) modulates migration of both neural crest and CM cells (19, 30). GalTase catalyzes the transfer of Gal from the sugar nucleotide (UDPGal) to terminal N-acetylglucosamine (GlcNAc) residues of glycoproteins, especially laminin.

A collagen culture system can mimic CM cell migration (5, 37). When cushion tissues were transplanted onto collagen type-I gel and incubated with medium, CM cells start to migrate in the gel. We recently reported that medium containing chicken serum strongly promotes migration of chick CM cells compared with medium containing calf serum (44). This finding indicates that chicken serum contains promoters for migration of chick CM cells.

If migration promoters in serum are glycoproteins, their function might be sensitive to lectin binding. Lectins themselves are also glycoproteins, and they bind to terminal carbohydrates with high specificity and affinity; for instance, wheat germ agglutinin (WGA) binds to terminal N-acetylneuraminic acid (NeuNAc) and GlcNAc. Thus, if some of the migration promoters have terminal GlcNAc, and if cell surface GalTase is responsible for migration, WGA should affect migration of CM cells. In other words, study lectins that inhibit CM cell migration should yield information concerning the possible role of sugar residues in migration.

To search for unknown glycoproteins promoting CM cell migration, we designed a migration inhibition assay with five lectins *in vitro* as the first step of the present study. By this screening, three kinds of lectins inhibited migration of CM cells. In order to check whether these lectins recognize migration promoting glycoproteins, we examined migration promotion activity of these lectin binding proteins.

MATERIALS AND METHODS

Fertilized Hi-brown eggs were incubated for six days at 37.5°C with humidified air. Embryos of stage 29 (18) were used. As we previously reported, the migration activities of CM cells of the CT cushion are higher than those in the AV cushion (44). We thus used CM cells of both the CT and AV cushions for the migration assay.

Migration inhibition assay with lectins. CT and AV cushions were removed from the heart in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo, Japan) including 10% heat inactivated chicken serum (Gibco Labs, Grand Island, NY, USA). Collagen gel culture was based on the method described by Bernanke and Markwald (5) or Runyan and Markwald (37). CT and AV cushions were transplanted on collagen type-I gel (2.4 mg/dish, Nitta Gelatin; Yao, Japan), and cultured with the same medium including serum at 37.5°C in 5% CO_2.

At 24 hr of culture, Concanavalin A (Con A), peanut agglutinin (PNA), soybean agglutinin (SBA), wheat germ agglutinin (WGA) from Honen Corporation (Tokyo, Japan) or pisum sativum agglutinin (PSA) from Sigma Chemical Company (St. Louis, MO, USA) was added to the medium at 100 μg /ml of final concentration.

Control lectins were prepared as follows. PSA and Con A (0.9 μM) was preincubated with 0.1 M α-D-mannose (Man), and a mixture of 0.1 M α-D-Man and α-D-glucose (Glc), respectively at 37.5°C for 6 hr. WGA (2.7 μM) was preincubated with 0.1 M α-D-GlcNAc and NeuNAc.

Migration inhibition assay with monosaccharides. As described for migration inhibition assay with lectins, inhibition by monosaccharides was tested by addition of competitors, α-D-Man, α-D-GlcNAc or NeuNAc, at a final concentration of 3 mg/ml.

Elution of lectin-binding proteins. Lectin-binding proteins were eluted from heat inactivated chicken serum with lectin affinity columns. Con A-, PSA- and WGA-agarose (Honen Co. Tokyo, Japan) columns were prepared with 5 ml of bed volume. These columns were washed with 50 ml of 0.1 M phosphate buffered saline containing 2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS). Chicken serum (20 ml) diluted with 20 ml of PBS was applied to the columns. After washing the columns with 250 ml of PBS, lectin-binding proteins were eluted with appropriate monosaccharides. For elution of Con A- and PSA-binding proteins, the elution buffer contained α-D-Man and α-D-Glc 100 mg/ml each. For elution of WGA-binding proteins, the elution solution contained α-D-GlcNAc and NeuNAc 100 mg/ml each.

Migration stimulation assay with lectin-binding proteins. Lectin-binding proteins with elution buffers (approximately 2 ml) were dialyzed with PBS for 12 hr. Protein concentration was measured with a protein assay kit (Bio-Rad, Richmond, CA, USA). The CT and AV cushions were cultured with the medium without serum but supplemented with Con A-, PSA- or WGA-binding proteins at final concentrations of 20 μg/ml to 200 μg/ml for 48 hr at 37.5°C in 5% CO_2.

Measurement of migration distance. The method of measurement of migration distance has been reported in a previous paper (44). Briefly, the transplanted tissues and migrating mes-

enchymal cells at 48 hr of culture were fixed with 4% formaldehyde in 0.1 M phosphate buffer. The tissues and cells were photographed within 1 hr after fixation. On the montaged photoprints, a line of the outermost migrating cells and rim of the tissue was digitized with a digitizer (KD4300 Graphtech Corp., Kobe, Japan), and the mean distance of migration was computed from the value of the area of migration with a personal computer (PC-386 VR, Seiko-Epson Corp., Suwa, Japan) and a personally designed BASIC program. The sample numbers in each experimental groups were 40 to 60 (approximately 1250 embryos in total). Variances in migration distances among experimental groups were evaluated by F-test. In most cases, variances between certain two experimental groups were not significantly different at a 5% significance level, hence the difference of migration distance was tested by ordinary *t*-test.

RESULTS

Migration inhibition assay with lectins. CM cells of both CT and AV cushions migrated more than 1,000 μm at 48 hr of culture without lectins (Fig. 1). Migration distance of CM cells of the CT cushion was greater than that of the AV cushion. PSA, Con A and WGA inhibited migration of CM cells of both CT and AV cushions ($p<0.01$; Fig. 1). Migration distances of CM cells with PSA and WGA were about 1/2 to 1/4 of that of the controls. In the present concentration, however, PNA and SBA did not affect migration of CM cells ($P>0.05$; Fig. 1). Twice the concentration (200 μg/ml) of PNA also did not affect migration of CM cells (data not shown).

WGA inhibited migration in a concentration dependent manner (Fig. 2). The concentration of WGA that reduces 50% migration distance of CM cells was estimated as 20 to 30 μg/ml. We did not examine concentration dependency of migration inhibitory effects of PSA and Con A.

The inhibition of migration by Con A and WGA was not due to the inhibition of collagen matrix function. Lectin Western blotting showed that Con A and WGA

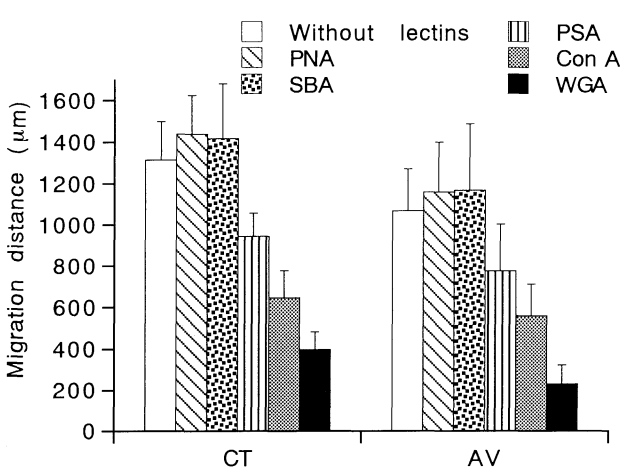

Fig. 1. Effects of lectins on chick CM cell migration. PSA, Con A, and WGA inhibited migration, but PNA and SBA did not. Migration distance of CM cells was reduced to less than 1/3 by WGA. CT: CM cells of the conotruncal cushion, AV: CM cells of the atrioventricular cushion.

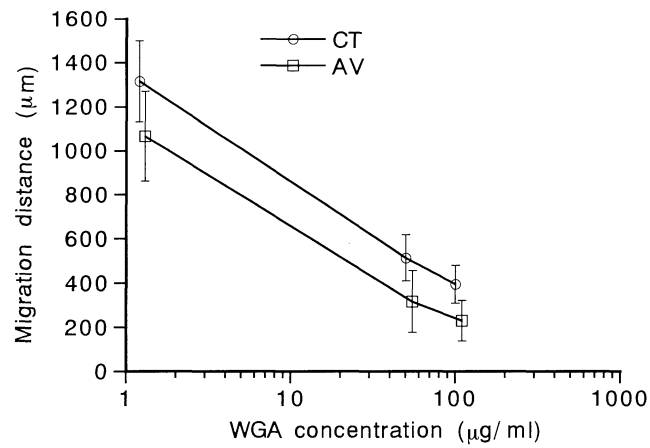

Fig. 2. Concentration dependency of migration inhibition by WGA. CT: CM cells of the conotruncal cushion, AV: CM cells of the atrioventricular cushion.

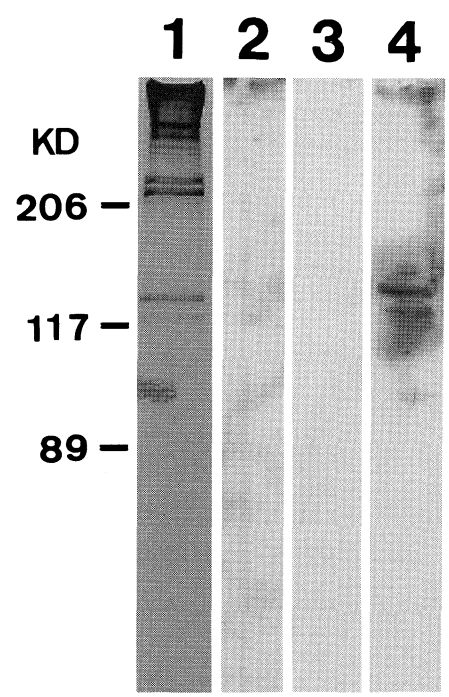

Fig. 3. Western blotting of collagen type-I with Con A, WGA and PSA. Con A and WGA did not bind to collagen type-I. PSA bound alpha chain of collagen type-I. Lane 1: SDS-PAGE of collagen type-I, Lane 2: with Con A, Lane 3: with WGA: Lane 4 with PSA.

did not bind to collagen type-I, whereas PSA bound to the alpha chain (Fig. 3).

Lectins preincubated with their specific binding monosaccharides still inhibited migration of CM cells. Preincubation with GlcNAc and NeuNAc clearly reduced the migration inhibitory effect of WGA ($p<0.01$), while almost no reduction of migration inhibitory effect of PSA was gained by preincubation with Man ($p>0.05$; Fig. 4). Migration distance of CM cells cultured with control-WGA was still lower than that without lectins (Fig. 4). Preincubation with Man and Glc enhanced the migration inhibitory effect of Con A ($p<0.05$; Fig. 4).

Migration inhibition assay with monosaccharides. If terminal Man, GlcNAc and NeuNAc are responsible for cell recognition and/or attachment, their addition into the culture medium possibly perturbs CM cell migration *in vitro*. With treatment of NeuNAc, CM cell migration was inhibited ($p<0.01$; Fig. 5). Migration inhibition by NeuNAc was at a similar level with that of WGA. Man and GlcNAc, however, did not inhibit CM cell migration ($p>0.05$; Fig. 5).

Migration stimulation assay with lectin-binding proteins. A large number of proteins of molecular weight ranging from 1-200 kD were eluted from the Con A- or WGA-agarose affinity columns (Fig. 6). In contrast, fewer proteins of 5 to 90 kD molecular weight were eluted from the PSA-agarose columns (Fig. 6).

WGA-, Con A- and PSA-binding proteins promoted CM cell migration in a concentration dependent manner *in vitro* (Fig. 7, 8, 9). At the 200 μg/ml concentration of Con A- and WGA-binding proteins, the migration distance of CM cells was significantly greater

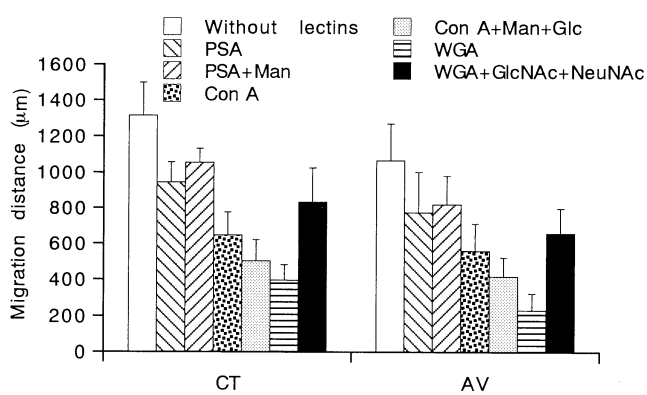

Fig. 4. Effects of preincubation with binding sugars on inhibition by lectins. Preincubation of WGA with α-D-GlcNAc and NeuNAc (control-WGA) dramatically reduced migration inhibition ability of WGA, while control-WGA still inhibited migration of CM cells. Preincubation of PSA with α-D-Man and α-D-Glc slightly reduced the migration-inhibition ability of PSA. Preincubation of Con A with Man and Glc inhibited migration more severely than did Con A alone. CT: CM cells of the conotruncal cushion, AV: CM cells of the atrioventricular cushion.

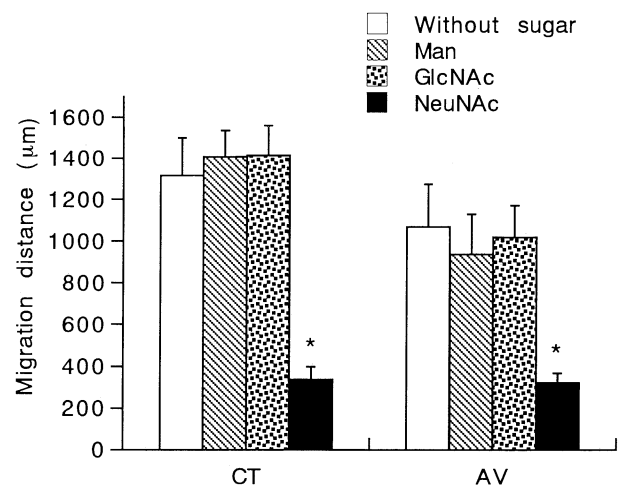

Fig. 5. Effects of monosaccharides on migration of CM cells. NeuNAc (3 mg/ml) severely inhibited migration of CM cells. At the same concentration, α-D-Man and α-D-GlcNAc did not inhibit migration. CT: CM cells of the conotruncal cushion, AV: CM cells of the atrioventricular cushion.

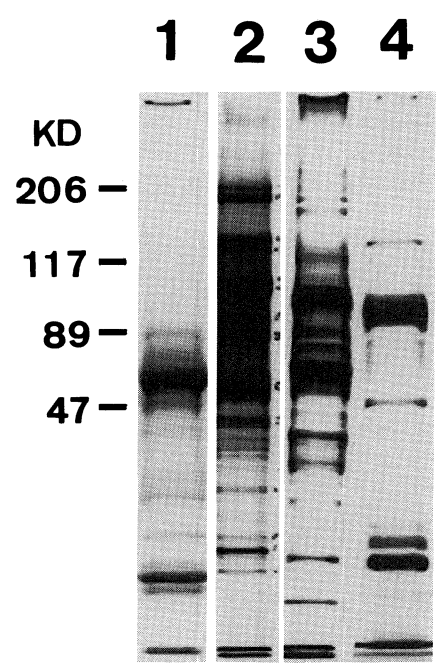

Fig. 6. SDS-PAGE of Con A-, WGA- and PSA-binding proteins eluted from affinity columns. Con A- and PSA-binding proteins was eluted with solution containing α-D-Man and α-D-Glc. For elution of WGA-binding proteins, α-D-GlcNAc and NeuNAc were used. Con A- and WGA-binding proteins consisted of proteins with widely variable range (1 to 200 KD) of molecular weights. PSA-binding proteins consisted of more limited proteins (5 to 90 KD). Lane 1: Chicken serum, Lane 2: WGA-binding proteins, Lane 3: Con A-binding proteins, Lane 4: PSA-binding proteins.

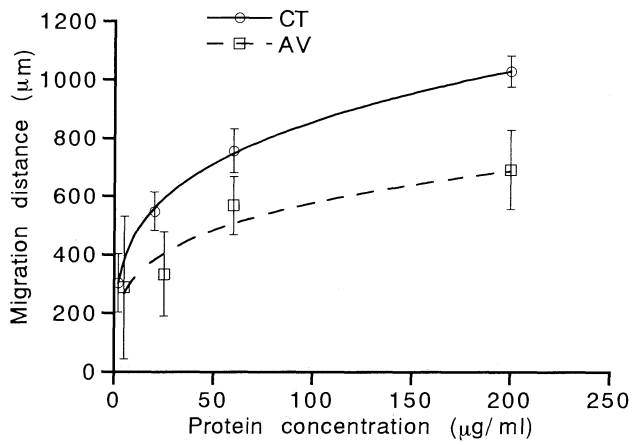

Fig. 7. Promoting activity of WGA-binding proteins on CM cell migration. The activity was concentration dependent with a high correlation coefficient. Regression was presented as follows. CT: $y=250.71 \times x^{0.26683}$ (R=0.98102), AV: $y=177.12 \times x^{0.25618}$ (R=0.95811). y: Migration distance, x: Protein concentration. CT: CM cells of the conotruncal cushion, AV: CM cells of the atrioventricular cushion.

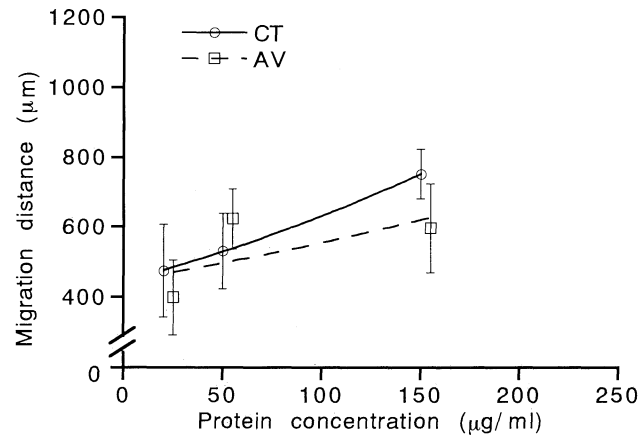

Fig. 9. Promoting activity of PSA-binding proteins on CM cell migration. At comparable concentrations, promoting activity of PSA-binding proteins was weaker than that of WGA- or Con A-binding proteins. CT: $y=443.77 \times e^{0.0035306x}$ (R=0.99992), AV: $y=445.04 \times e^{0.002214x}$ (R=0.57505). y: Migration distance, x: Protein concentration. CT: CM cells of the conotruncal cushion, AV: CM cells of the atrioventricular cushion.

($p<0.05$) than that without the proteins. The migration promoting activity of 330–365 μg/ml of Con A-binding proteins or 500–1100 μg/ml of WGA-binding proteins was estimated as equivalent to the migration promoting activity of 10% (v/v) heat-inactived chicken serum in the medium.

DISCUSSION

Chick embryonic CM cells show high migration activity in hydrated collagen lattice culture (5, 37). We previously reported that chicken serum dramatically promotes migration of chick embryonic CM cells compared with calf serum (44). This finding indicates that chicken serum contains factor(s) that promote chick CM cell migration. In the present study, Con A, WGA or PSA inhibited migration of chick CM cells stimulated by chicken serum. These lectins might bind migration related molecules in chicken serum, and inhibit their function.

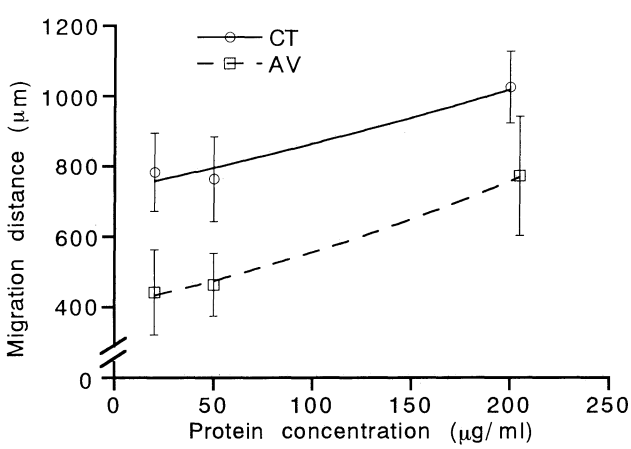

Fig. 8. Promoting activity of Con A-binding proteins on CM cell migration. Promotion of migration by Con A was also concentration dependent. CT: $y=732.62 \times e^{0.0016516x}$ (R=0.9791), AV: $y=407.45 \times e^{0.0031037x}$ (R=0.99837). y: Migration distance, x: Protein concentration. CT: CM cells of the conotruncal cushion, AV: CM cells of the atrioventricular cushion.

Results of migration inhibition assay with lectins suggest that some of the migration relating molecules are glycoconjugates such as glycoproteins and glycosaminoglycans, or their complexes, although glycosaminoglycans occur chiefly in the extracellular matrices rather than serum (20, 21). The migration-inhibiting ability of WGA was dramatically reduced by preincubation with α-D-Man and NeuNAc. Furthermore, WGA-binding proteins eluted from a WGA-affinity column with the elution buffer containing α-D-GlcNac and NeuNAc promoted migration of CM cells with distinct dependence upon concentration ($R>0.95$). These results suggest not only the existence of WGA-binding migration promoters in chicken serum, but also that some migration promoters in chicken serum have the terminal GlcNAc and/or NeuNAc residues.

Con A, that binds to Man and Glc residues, inhibited migration of CM cells. Con A preincubated with α-D-Man and α-D-Glc, however, did not reduce inhibition ability. In spite of this fact, we have evidence for the presence of the terminal Man and/or Glc on the migration promoter in chicken serum. From chicken serum, Con A-binding proteins which promoted migration of CM

cells, were eluted from the Con A-affinity column with the elution buffer including α-D-Man and α-D-Glc. Furthermore, the migration promotion of Con A-binding proteins was concentration dependent. A high correlation was indicated between concentration of Con A-binding proteins and migration distance of CM cells ($R > 0.97$). These facts thus strongly suggest that some proteins in chicken serum which have the terminal Man and/or Glc residues are migration promoting factors. Con A might inhibit migration of CM cells with other mechanisms rather than binding to the terminal Man and/or Glc of migration promoter. For instance, Con A binds also to GlcNAc (49). In any case, the terminal Man and Glc were not functionally essential in CM cell migration, because additional Man and Glc in the medium did not disturb migration.

Others have shown that glycoproteins mediate CM cell migration (29, 35). Many glycoproteins mediating cell migration have a common cell attachment site, present as the RGD sequence, in their amino acid chain (46). Findings of migration inhibition assay by the synthetic peptides, however, showed that mediation of CM cell migration involves more than the RGD sequence alone (10, 26). Instead of certain amino sequences, the terminal sugar residues may play an important role in CM cell migration. It has been reported that cell surface GalTase promotes cell migration (19, 30, 38), while cells with overexpressed cell surface GalTase by gene transduction become stationary (3). GalTase recognizes terminal GlcNAc residue (39). These facts suggest that the terminal GlcNAc residue of extracellular glycoproteins and/or proteoglycans are, at least partially, responsible for promotion of CM cell migration (30). In general, it has been thought that cell surface GalTase interacts predominantly with the GlcNAc residue of laminin (6, 19). Laminin is a major component of the cardiac jelly through which CM cells migrate in the developing heart (10, 29). Inhibition by WGA of CM cell migration might plausibly indicate the relation of the terminal GlcNAc residues of serum promoters with CM cell migration. The results of the present study, however, would suggest less mediation of GalTase with the serum promoters in chick CM cell migration, because extra GlcNAc did not disturb migration.

On the other hand, lectins might inhibit the function of surface receptors mediating cell migration. Terminal sugar residues of cell surface molecules are possibly involved in cell motility (33). Recognition and interaction of some C-type lectins with extracellular adhesion proteins could be carbohydrate mediated (1). In fact, inhibition of the glycosylation of the integrin molecules reduced the affinity to ligands (2), whereas integrins recognize and interact with certain amino sequences of extracellular adhesion molecules (16, 23, 47), and the sugar chain of integrins has not been speculated essential part in the interaction with ligands (28). In the migration inhibition assay of the present study, lectins could bind to cell surface carbohydrates and block their function in relation to cell migration. At least, the molecular size of lectins is possibly enough to mask the ligand recognition site of cell surface receptors when lectins bind to the terminal sugar residues of the receptor molecules. Thus, it can be said that the inhibition of the function of migration-relating cell surface receptors by lectins is not a negligible factor in the interpretation of the migration inhibition assay with lectins.

In the monosaccharides used in the present study, NeuNAc clearly inhibited migration of chicken CM cells. NeuNAc is one of the most common terminal residues, and it is widely distributed in the tissue (41). The terminal NeuNAc has been thought to play an important role in cell attachment and recognition, although the residue itself might not be a direct interacting site of the molecules. For instance, neural cell adhesion molecules (N-CAM) is highly sialylated (4, 48). Binding of N-CAM is diminished by the terminal NeuNAc residues (4, 9), probably by reason that the terminal NeuNAc residues mask or change conformation of binding sites of their amino-terminal domains (7). Extra NeuNAc might mask binding domains of migration relating molecules or disturb the binding control of cell surface receptors by cations (16, 24) through its negative charging. On the other hand, it cannot be clearly stated that the terminal NeuNAc is not a functional site of migration promoters in serum. In fact, cell surface P-selectin does recognize and bind to the terminal NeuNAc residue of glycoproteins (11). Further information will be necessary to clarify whether the terminal NeuNAc residue of extracellular molecules is essential in mediating cell migration.

Effects of collagen type-I on cell migration should also be focused. PSA bound to collagen type-I (see Fig. 3), suggesting that PSA might inhibit possible mediation of collagen matrix in CM cell migration. Our preliminary study, however, showed that collagen type-I did not significantly enhance migration promotion by chicken serum proteins (unpublished data; Sumida *et al.*, J. Mol. Cell Cardiol. 27: A82, 1995, abstract). Further study of migration inhibition of PSA and binding of PSA to collagen type-I is in progress.

Some findings have suggested that the terminal sugar residues of migration-related molecules for CM cells were different among species (12, 13). In the present study, PNA did not inhibit CM cell migration. Lectin histochemistry also showed that PNA-binding factors were not abundant in the conotruncal and atrioventricular cushions in chicks (13). In rat embryos, however, the conotruncal and atrioventricular cushions were highly positive with PNA-staining (13). Thus, PNA may inhibit CM cell migration in rats. The sugar chain of migration-related molecules may be widely variable and differ-

ent among species. Although we do not know whether variation in terminal residues indicate functional difference among molecules, the mechanisms of CM cell migration may be different among species (44, 45).

The present study brings new information concerning the activity of terminal sugar residues of migration promoters in chicken serum, while a large variety of molecules may participate in the mediation of CM cell migration. Some of these promoters might be well known molecules. Our unpublished study (Sumida *et al.*, J. Mol. Cell. Cardiol. 27: A82, 1995, abstract) presented that the RGD synthetic peptide partially inhibited migration promotion by Con A-binding proteins of chicken serum. We believe that the present study suggests the existence of novel migration promoting molecules in serum in addition to those molecules interacting with the RGD amino-sequence or interacting with cell surface GalTase.

Acknowledgement. The authors thank Dr. M. Hoshi, Research Institute for Radiation Biology and Medicine, Hiroshima University, for his helpful experimental support.

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(Received for publication, February 26, 1997
and in revised form, April 4, 1997)