Chondrocyte Migration to Fibronectin, Type I Collagen, and Type II Collagen

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ABSTRACT. It is well known that cellular interactions, such as cell adhesion, migration, invasion, between cells and the extracellular matrix are mediated by the integrin family of cell surface receptors. Chondrocytes are surrounded by an abundant extracellular matrix, but there is less information on the cellular receptors which interact with this matrix. In our studies, fibronectin, type I collagen, and type II collagen promoted haptotactic and chemotactic migration of chondrocytes, as determined using a modified Boyden chamber system. Treatment of chondrocytes with tyrosine kinase inhibitor, herbimycin or genistein, resulted in a dose dependent inhibition of migration toward these matrix proteins, whereas adhesion of chondrocytes was not influenced. This indicated the existence of functional relationships between protein tyrosine phosphorylation and chondrocyte migration following the adhesion of chondrocytes to matrix proteins. Further study showed that the peptide GRGDSP inhibited chondrocyte migration to fibronectin but not to collagen. On the other hand, chondrocytes migrated toward the tetra-RGD containing peptide, but not the peptide GRGDSP, in a dose dependent fashion. These observations suggest that cross-linking or clustering of integrins is essential to induce transmembrane signaling related to tyrosine phosphorylation for chondrocyte migration toward fibronectin.

Chondrocytes are responsible cells for the mainten ance of the extracellular matrix (ECM) in cartilage tissue, which contains a variety of proteins, such as cartilage-specific proteoglycan aggrecan (1), type II collagen (2), fibronectin (3, 4), tenascin (5), and several minor collagens (6–10). Cartilage ECM is reconfigurable, and the tissue expands when the chondrocytes divide and se crete more matrix. As chondrogenesis proceeds, type I collagen and large chondroitin sulfate proteoglycan (designated PG-M) are replaced by type II collagen and the proteoglycan aggrecan (11, 12), which are known as typical markers for cartilage tissue. This dynamic change in the components of ECM proteins during chondrogenesis indicates that the ECM may play an important role in the control of chondrocyte differentiation.

Recent studies have shown that chondrocytes are capable of attaching to a number of ECM proteins and that the integrin family of ECM receptors is implicated in chondrocyte adhesion to ECM proteins (13–15). Enomoto et al. (16) demonstrated that β1 integrins mediate chondrocytes adhesion to type I collagen, type II collagen, and fibronectin. In many types of cells, integrin-mediated linkage of the ECM to the cytoskeleton occurs at sites known as focal adhesion (17). These sites are considered not only to have a structural function, also to serve as sites for initiating signal transduction from the ECM to the interior of the cells. Binding of adhesive ligands to integrins can induce protein tyrosine phosphorylation in fibroblasts, carcinoma cells, T lymphocytes, B cells, platelets, monocytes, and neutrophils (18–25). Several studies concerning the capacity of signal transduction via β1 integrins have been published recently (19, 25–30). In analogy, it is therefore possible that the binding of certain ligands, such as fibronectin, to β1 integrins results in transduction of a signal. One important function of this signal the transduction via integrins is the ability to direct cell movement. These studies have shown that ECM components promote the directed migration in vitro of various cells. However, there is less information about the cellular receptors which interact with ECM in the cartilage tissue.

Here we present the first evidence to show that fibro nectin, type I collagen, and type II collagen promote haptotactic and chemotactic migration of chondrocytes, as determined using a modified Boyden chamber system. The studies using tyrosine kinase inhibitors show that the relationship is important between protein tyrosine phosphorylation and chondrocyte migration following the adhesion of chondrocytes to ECM proteins. In addition, the further studies using two kinds of RGD-containing peptides suggest that cross-linking or clustering of β1 integrins is necessary to induce transmembrane signaling for chondrocyte migration toward
fibronectin.

**MATERIALS AND METHODS**

*Materials.* Human plasma fibronectin was purified by affinity chromatography on gelatin Sepharose as described elsewhere (31). Type I collagen (fetal bovine skin) and type II collagen (bovine sternum) were purchased from Chemicon International Inc. (Temecula, CA). Herbimycin and genistein were purchased from Calbiochem-Novabiochem Co. (La Jolla, CA). The GRGDSP peptide was obtained from Peninsula Labs. (Belmont, CA). The tetra-GRGDSP peptide, CGRGDSPGRGDSPGRGDSPC, which has a closed ring through the cysteine residues, was synthesized on an Applied Biosystems 431A peptide synthesizer and purified as described previously (32).

*Cell culture.* Chondrocytes were isolated from articular cartilage of New Zealand White rabbits (33). Cells were cultured as high-density monolayers (2×10⁵ cells/cm²) in DMEM containing 10% fetal bovine serum (FBS) and antibiotic supplements with the media changed every 48 hours for 5 days (31).

*Cell attachment assays.* The cell attachment assays were performed as previously described (35). In brief, microtiter plates (Becton Dickinson Labware, Franklin Lakes, New Jersey), coated overnight with the proteins, were washed with PBS and unoccupied binding sites were blocked with 1% bovine serum albumin (BSA). Cells were harvested from cell culture flask with collagenase (Boehringer Mannheim, Germany), washed with DMEM containing 10% FBS three times and then washed once with serum-free media (DMEM containing 1% BSA). Cells were counted and diluted to 1×10⁶ cells /ml in serum-free media. Cell suspension (100 μl) was placed in each coated well and allowed to incubate for 45 min at 37°C. Unattached cells were washed out and attached cells were fixed with 10% formalin. Cells stained with 1% toluidine blue were then counted using an inverted microscope. Cells from at least three microscope fields were counted and the mean and SD of adherent cells were determined.

*Cell migration assays.* Chemotaxis was assayed using 24-well tissue culture plates, and cell culture inserts containing polyethylene terephthalate membranes, 8 μm pore size (Becton Dickinson Labware) as previously described (36). Triplicates of lower wells were filled with different concentrations of fibronectin, type I collagen, or type II collagen diluted in DMEM containing 1 mg/ml BSA, whereas the upper wells were filled with 4×10⁵ cells/200 μl of DMEM containing 1 mg /ml BSA. The chambers were incubated for 5 hours in a humidified incubator at 37°C. After performing the incubation, the membranes were removed, and then fixed with methanol, stained with hematoxylin and eosin. The membranes were subsequently placed onto glass slides and remaining cells on the upper side of the membrane were wiped off. The number of migrated cells were counted by light microscopy at 200-fold magnification. For each triplicate, the number of cells in nine microscope fields was determined and the mean and SD were calculated.

Haptotactic assays were also performed as previously described (36). The membranes of the upper wells were coated with different concentrations of ECM proteins diluted in PBS overnight at 4°C. The membranes were extensively washed with PBS and then with distilled water. The membranes were subsequently air dried. The lower wells of the 24-well Boyden chamber were filled with DMEM containing 1 mg/ml BSA. The upper wells precoated with ECM proteins were filled with 4×10⁵ cells/200 μl of DMEM containing 1 mg/ml BSA and chondrocyte migration to the underside of the membrane was measured after incubation. Incubation, fixing, and staining of the membranes were performed as described above.

**RESULTS**

*Fibronectin, Type I Collagen, and Type II Collagen*

![Fig. 1. Chondrocyte migration assay in the Boyden chamber system. Migrated chondrocytes on the membrane coated with 5 μg/ml fibronectin (upper) and non-migrated chondrocytes on the membrane coated with 1 mg/ml BSA as negative control (lower).](image-url)
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Fig. 2. Haptotactic and chemotactic migration of chondrocytes to different concentrations of ECM proteins in the Boyden chamber system. Rabbit articular chondrocytes were assayed for haptotactic and chemotactic migration toward fibronectin (A, B), type I collagen (C, D), and type II collagen (E, F). The number of cells migrating to a protein concentration of 0 μg represents migration on membranes coated with BSA 1 mg/ml (haptotaxis) or migrating toward BSA 1 mg/ml in the lower compartment (chemotaxis). Note the different scales of the y-axes for different proteins.
**Promote chondrocyte Migration.** Migratory chondrocytes on a membrane coated with 5 μg/ml fibronectin and non-migratory chondrocytes on a membrane coated with 1 mg/ml BSA as negative control are shown (Fig. 1). The cells on the lower side of the membrane were fixed and stained as described in Materials and Methods. As shown in Fig. 2, fibronectin, type I collagen, and type II collagen promoted chondrocyte migration, and coating with proteins (haptotaxis) was in general more efficient in promoting directed motility than the same protein in solution (chemotaxis). For each protein, increasing the gradient resulted in increased number of migrating cells until a plateau was reached. In the case of fibronectin, haptotaxis as well as chemotactic migration reached a plateau at a concentration of 10 μg/ml, whereafter no or only slight increase of migration could be observed. Type I collagen and type II collagen increased haptotactic and chemotactic migration of chondrocytes at concentrations from 0 to 40 μg/ml. In all subsequent experiments with these proteins as in attractant and migration assays, 10 μg/ml fibronectin, 50 μg/ml type I collagen, and 50 μg /ml type II collagen were used.

**Herbimycin and Genistein Inhibit Chondrocyte Migration to Fibronectin, Type I Collagen, and Type II Collagen.** The migration of chondrocytes caused by ECM proteins suggests that there may be functional relationships between protein tyrosine phosphorylation during transmembrane signaling and cell migration. This possibility was tested by the use of tyrosine kinase inhibitors. Herbimycin and genistein have been used to inhibit the signal pathway mediated by protein tyrosine kinases (37, 38). Pretreatment of chondrocytes with herbimycin for 1 hour resulted in a dose-dependent inhibition of cell migration (Fig. 3A). At 20 μM, herbimycin strongly suppressed the migration promoted by ECM proteins. The inhibitory effects of genistein were noticeable at 50 μM (Fig. 3B). Neither herbimycin nor genistein interfered with the adhesion of chondrocytes to fibronectin, type I collagen, and type II collagen. These results indicate that tyrosine phosphorylation plays critical roles in chondrocyte migration (Table I).

**A GRGDSP Peptide Inhibits Chondrocyte Migration to Fibronectin.** The peptide, GRGDSP, at 1 mM concentration, has been shown to inhibit migration of chondrocytes to fibronectin (25% of control) while migration to type I collagen and type II collagen was not influenced by this peptide (Fig. 4). This suggests that chondrocyte migration needs binding to the RGD sequence of fibronectin mediated by β1 integrin in a first vital part of the migratory response.

**Cross-linking or Clustering of β1 Integrins Induces Chondrocyte Migration.** To further analyze the role of β1 in chondrocyte migration, we examined the possibility of chemotactic migration promoted by the RGD sequence using two kinds of peptides, the GRGDSP and tetra-GRGDSP peptides. Ligation of integrins with the tetra-GRGDSP peptide resulted in chondrocyte migration in a dose-dependent fashion, while the GRGDSP peptide had no effect (Fig. 5). These observations suggest that cross-linking or clustering of integrins can produce signaling leading to chondrocyte migration because the GRGDSP peptide did not stimulate a response.

**DISCUSSION**

Cellular recognition of the ECM and of other cells
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Table I. Adhesion of chondrocytes to fibronectin, type I collagen, and type II collagen in the absence or presence of genistein and herbimycin.

<table>
<thead>
<tr>
<th>ECM proteins</th>
<th>Control (Mean ± SD)</th>
<th>Genistein 100 μM (Mean ± SD)</th>
<th>Herbimycin 50 μM (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fibronectin</td>
<td>112 ± 11</td>
<td>120 ± 15</td>
<td>109 ± 13</td>
</tr>
<tr>
<td>type I collagen</td>
<td>49 ± 8</td>
<td>46 ± 9</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>type II collagen</td>
<td>63 ± 8</td>
<td>65 ± 7</td>
<td>68 ± 10</td>
</tr>
</tbody>
</table>

*a The cells were pretreated with the different inhibitors for 60 min at 37°C before the cell attachment assay was performed as described in Materials and Methods. The wells of 96-well microtiter plates were coated with 50 μg/ml ECM proteins overnight at 4°C. The values represent mean and SD of the number of adherent cells of three microscope fields.

Fig. 4. The effect of GRGDSP peptide on haptotactic migration of chondrocytes toward ECM proteins. Chondrocyte migration toward fibronectin (10 μg/ml), type I collagen (50 μg/ml), and type II collagen (50 μg/ml) was assayed by using the Boyden chamber system in the presence of different concentrations of GRGDSP peptide.

Fig. 5. Chemotactic migration of chondrocytes toward fibronectin and RGD-containing peptides. Chondrocytes were assayed for chemotactic migration toward fibronectin and two kinds of RGD-containing peptides, GRGDSP and tetra-GRGDSP (tGRGDSP), in the Boyden chamber system.

Our study demonstrated that ECM proteins promote haptotactic and chemotactic motility of chondrocytes. The inhibition assay using herbimycin and genistein showed the functional relationships between protein tyrosine phosphorylation during transmembrane signaling and chondrocyte migration. Since the β1 subfamily of integrins in considered to be primarily responsible for the adherence of chondrocytes to fibronectin, type I collagen, and type II collagen, the signaling pathway leading chondrocyte migration may occur by integrin-protein ligation.

Four different β1 integrins, α3β1 (44), α4β1 (45–47), α5β1 (48, 49), and αvβ1 (50), have been shown to be receptors for fibronectin. Taking together its presence in chondrocytes, sensitivity to inhibition by RGD peptides (16), divalent cation requirements (51), and localization in adhesion plaques, α5β1 appears to function as the ma-
jor fibronectin receptor for chondrocytes. In our studies, chondrocyte migration to fibronectin but not to collagens was inhibited by the linear peptide GRGDSP. It has been demonstrated that linear RGD-containing peptide such as GRGDSP did not inhibit cell attachment to collagen, whereas cyclic RGD-containing peptide, CGRGDSPC, was capable of inhibiting it (52). This indicates that the conformation of the RGD sequence may be important in cell binding to collagen mediated by α2β1 integrin. To investigate the ability of RGD peptides such as GRGDSP did not inhibit cell attachment major fibronectin receptor for chondrocytes. In our studies, chondrocyte migration to ECM proteins. Our results strongly suggested that the induction of tyrosine phosphorylation resulting in chondrocyte migration was mediated through cross-linking or clustering of β1 integrins could in-

Here we have demonstrated that integrin-mediated chondrocyte migration to ECM proteins. Our results strongly suggested that the induction of tyrosine phosphorylation resulting in chondrocyte migration was mediated through cross-linking or clustering of integrins.

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