Integrin expression in cells of the intervertebral disc

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Abstract

In this study, we investigated the profile of integrin expression in human and porcine intervertebral disc tissue. Differences in extracellular matrix composition between anulus fibrosus (AF) and nucleus pulposus (NP) regions of the disc, as well as differences in cellular responses to environmental stimuli, suggest a role for integrins in presenting matrix signals that may mediate these responses. Human disc tissue and porcine AF and NP tissue were stained with antibodies to alpha integrin subunits 1–6, V and IIb, and beta integrin subunits 1–6 and graded for evidence of positive staining on a scale from 0 (no staining) to 3 (high incidence of staining). Human tissue expressed α and β integrin subunits shown to be present in articular cartilage, including α_1, α_5 and α_V. Porcine AF tissue expressed similar integrin subunits to human disc, with both expressing α_1, α_5, β_1, β_3 and β_5 subunits, whereas porcine NP tissue expressed higher levels of α_6, β_1 and β_4 than AF tissue. The expressed subunits are known to interact with proteins including collagens, fibronectin and laminin; however, additional studies will be required to characterize the interactions of the integrin subunits with specific matrix constituents, as well as their specific involvement in regulating environmental stimuli.

Key words anulus fibrosus; cell–matrix interaction; human; nucleus pulposus; porcine.

Introduction

Integrins are a class of cell adhesion molecules that regulate interactions between a cell and its surrounding matrix. One alpha (α) and one beta (β) subunit associate non-covalently to form each functional integrin. To date, eight β and 18 α subunits are known to exist in humans, which are known to form only 24 distinct heterodimers. Integrins participate in signalling events in many cell types (Hynes, 2002), controlling gene expression (Hynes, 1992; Danen & Yamada, 2001; Takahashi et al. 2003), cell cycle (Gumbiner, 1996; Segat et al. 2002; Watt, 2002) and apoptosis (Bates et al. 1995; Matter & Ruoslahti, 2001; Lewis et al. 2002; Miranti & Brugge, 2002). Cells of cartilaginous tissues exist in an avascular and alymphatic environment, where they are surrounded by considerable amounts of extracellular matrix that provides important signals to mediate cell differentiation, survival and metabolism (Loeser, 2002). Loeser and co-workers have documented the presence of many α and β integrin subunits in articular cartilage, and demonstrated their importance in presenting matrix signals to chondrocytes from relevant proteins, including collagens and fibronectin (Loeser, 1993). Human articular chondrocytes may use $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{10}\beta_1$ integrins to interact with type II collagen, and $\alpha_1\beta_1$ and $\alpha_2\beta_1$ may also mediate adhesion to type VI collagen and matrilin-1 (Camper et al. 1998; Loeser et al. 2000). The $\alpha_5\beta_1$ subunit is used ubiquitously by many cell types, including chondrocytes, to interact with fibronectin, along with the $\alpha_v\beta_3$ integrin, which may also be used for this interaction. Studies in chondrocytes have demonstrated the importance of matrix signals from fibronectin in regulating collagenase expression and in promoting cell survival (Forsyth et al. 2002; Loeser et al. 2003). Furthermore, studies have shown an up-regulation in integrin expression *in situ* in osteoarthritic cartilage compared with normal cartilage (Loeser et al. 1995), suggesting a role for integrins in regulating disease states in cartilage. Still other studies have shown that integrin expression changes as articular cartilage matures

from fetal cartilage to adult cartilage, indicating a role for integrins in chondrocyte proliferation and differentiation (Holmvall et al. 1995; Salter et al. 2001). Little is known of integrin expression patterns in cartilaginous tissues other than articular cartilage, however, nor their functional role in mediating cell metabolism or cell cycle.

The intervertebral disc is composed of two distinct cartilaginous tissues, the anulus fibrosus (AF) and the nucleus pulposus (NP). The AF is considered to be fibrocartilaginous, and to consist primarily of layers, or lamellae, composed of highly orientated fibres of types I and II collagen (Schollmeier et al. 2000). The NP contains a higher content of type II collagen that is randomly orientated, with a much higher concentration and polydispersity of proteoglycans. Cells of the AF regions have been shown to orientate along the predominant collagen fibre directions of the lamella, and to form elongated processes that suggest a strong physical interaction with the extracellular matrix (Errington et al. 1998; Bruehlmann et al. 2002; Baer et al. 2003). Cells of the innermost AF and NP regions appear more rounded and may accumulate significant pericellular matrix regions containing fibronectin, type VI and II collagens, and proteoglycans (Roberts et al. 1991, 1994). It is noteworthy that cells of the AF and NP regions respond differently to environmental stimuli, with evidence that AF fibrochondrocytic cells change their gene expression in response to physical stimuli such as compression or osmotic pressure, whereas cells of the NP do not respond in a similar manner to these same stimuli (Lotz et al. 2001; Chen et al. 2002, 2003; MacLean et al. 2003; Boyd et al. 2004). Differences in the composition of extracellular matrix between AF and NP regions, as well as the evident differences in cellular responses to environmental stimuli, suggest a role for integrins in presenting matrix signals that mediate cellular responses in the intervertebral disc.

The objective of this study was to characterize the profile of integrin subunit expression in human and porcine tissues of the intervertebral disc. A subgroup of integrins were selected for study because of their known interactions with proteins expressed in fibrocartilage and hyaline cartilage, including types I, II ($\alpha_1\beta_1$, $\alpha_2\beta_1$), VI ($\alpha_1\beta_1$) and XI collagens (α_3, α_6), and fibronectin (α_3, α_4, α_5, α_V). Results of this study reveal similarities in integrin expression between mature human and porcine AF tissues, as well as differences between porcine AF and NP tissues that may be key to understanding the extracellular matrix interactions that regulate their differential biological behaviours.

Materials and methods

Tissue isolation and sample preparation

Intervertebral disc tissues were obtained from three human patients (ages 42–50) undergoing surgery for transforaminal lumbar interbody fusion. One disc sample, largely encompassing the inner and outer anulus fibrosus, was obtained from lumbar level L4–5 or L5–S1 of each patient. All samples showed morphological evidence of moderate degeneration. Tissue specimens were washed in phosphate-buffered saline (PBS), equilibrated in 30% sucrose dissolved in PBS and then flash frozen in liquid nitrogen for cryosectioning. In the mature human disc, there is little demarcation between discrete zones of the fibrocartilaginous AF and NP tissues; thus, we undertook a study of skeletally immature porcine tissues in order to examine zonal differences in integrin expression. Intervertebral discs ($n = 2$ for each of three animals) were obtained from the lumbar region of skeletally immature porcine spines (3 months old, Nahunta Pork Outlet, Raleigh, NC, USA) and dissected into regions containing the AF and NP tissues. Tissue samples were immediately flash frozen in liquid nitrogen for cryosectioning.

Immunohistochemistry

Anti-human anti-integrin antibodies were purchased from Chemicon (Temecula, CA, USA) except anti-$\beta1$, which was purchased from Coulter Corporation (Miami, FL, USA). Seven-micrometre-thick sections for immunohistochemistry were fixed in 100% acetone. Sections were then washed in water, followed by PBS, before being incubated with 10% normal non-immune goat serum (Zymed, South San Francisco, CA, USA) for 2 h. Sections were then incubated with one of the following anti-integrin primary antibodies: rabbit polyclonal anti-α_1 (AB1934), mouse monoclonal anti-α_2 (MAB1950Z), anti-α_3 (MAB1952Z), anti-α_4 (MAB16983Z), rabbit polyclonal anti-α_5 (AB1928), mouse monoclonal anti-α_6 (CD149f), rat monoclonal anti-α_V (MAB1378), mouse monoclonal anti-α_{IIb} (MAB1990), mouse monoclonal anti-β_1 (4B4), anti-β_2 (MAB1414), rabbit polyclonal anti-β_3 (AB1932), anti-β_4 (AB1922), anti-β_5 (AB1926), or mouse monoclonal anti-β_6 (MAB2076Z). Antibodies against the α_1, α_5 and β_3
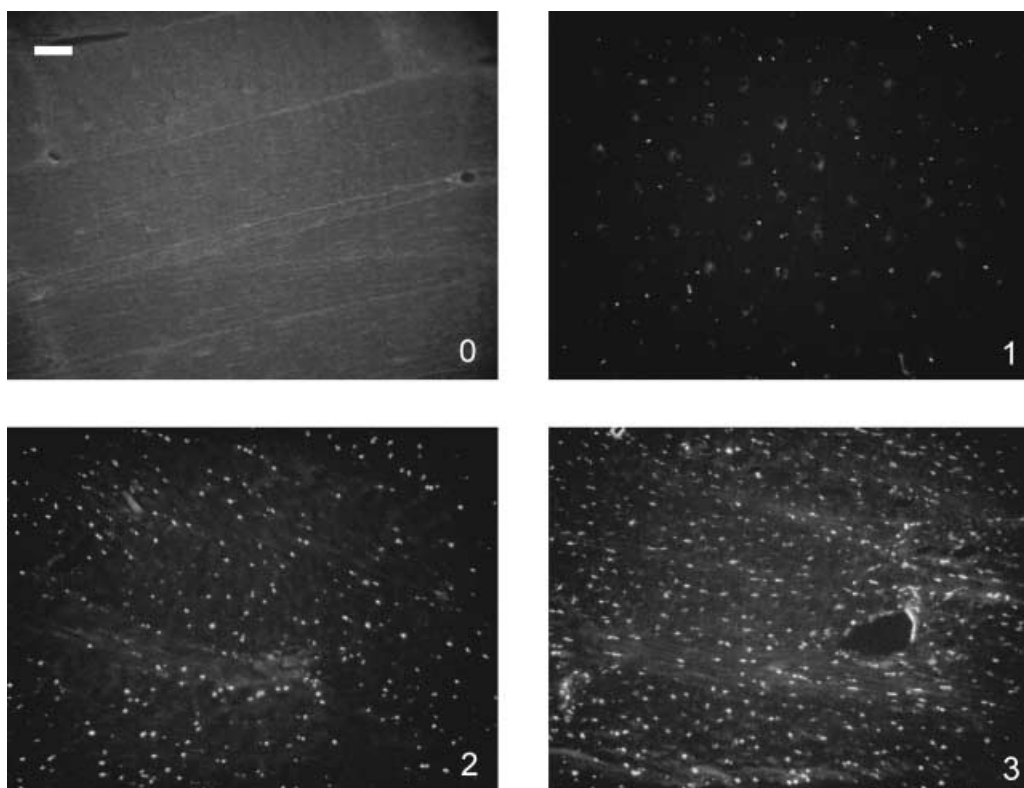
Fig. 1 Images of porcine AF sections stained for integrin subunits demonstrating the grading scale: (A) no staining, (B) low incidence of staining, (C) moderate incidence of staining, (D) high incidence of staining. Scale bar = 100 μm.

integrin subunits are known to cross-react with porcine tissues, whereas all other antibodies cross-reacted with porcine NP, AF or porcine meniscus tissue in preliminary screening. Control sections were incubated with an equal volume of non-immune goat serum. Following overnight incubation at 4 °C, sections were washed three times in PBS and then incubated with appropriate fluorescent secondary antibodies (Alexa Fluor® 488, Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Sections were then mounted with GVA mounting medium (Zymed) and immediately imaged on an inverted fluorescence microscope (Zeiss Axiovert S100, Thornwood, NY, USA).

Analysis

Each human disc, or porcine AF or NP, was treated as one sample. Two sections from each sample were analysed. From each section, five representative images were taken for each integrin subunit. NP cells from young discs were observed to exist in large clusters, often with membrane–membrane contacts, as has been previously described (Trout et al. 1982; Baer et al. 2003; Hunter et al. 2003), making it difficult to determine

cell boundaries, where integrin staining was expected to be seen. These observations were consistent with the pattern of integrin staining observed in the NP, which often followed the morphology of the entire cell cluster, rather then individual cells. Although it may have been possible to ascertain individual cell staining in the AF sections, the staining pattern observed in the NP made it difficult to ascertain staining for individual cells in this region. Because it was our aim to compare levels of integrin expression between AF and NP tissues, all sections were graded semi-quantitatively on a scale according to intensity of staining, as 0 (no staining), 1 (low incidence of staining), 2 (moderate incidence of staining) or 3 (high incidence of staining) (Fig. 1). Some background staining was observed in human and porcine AF sections due to collagen autofluorescence; however, images containing background staining were not graded based on background fluorescence, but rather on the number and intensity of punctate staining, as would be expected to be associated with integrins. A mean score was calculated for each integrin subunit based on all scores from all samples. Scores are reported as mean score ± standard deviation.

Table 1 Mean incidence of staining for (a) alpha subunits and (b) beta subunits in human disc, porcine NP and porcine AF tissues. Data are presented as mean ± SD

(a)	α_1	α_2	α_3	α_4	α_5	α_6	α_V	α_{IIb}
Human disc	0.8 ± 1.0†	0 ± 0*	0 ± 0*	0 ± 0*	1.7 ± 0.6‡	0 ± 0*	1.7 ± 0.5‡	0 ± 0*
Porcine AF	2.5 ± 0.7‡	0 ± 0*	0.2 ± 0.4*	0.1 ± 0.3*	2.1 ± 0.7‡	0.5 ± 0.7†	0.0 ± 0.2*	0.2 ± 0.4*
Porcine NP	2.6 ± 0.7‡	0.0 ± 0.6*	0.0 ± 1.0*	0.0 ± 0.2*	1.5 ± 0.6‡	2.2 ± 0.6‡	0.2 ± 0.4*	0.3 ± 0.5*

(b)	β_1	β_2	β_3	β_4	β_5	β_6
Human disc	1.2 ± 0.8†	0.2 ± 0.4*	1.5 ± 0.6†	0.3 ± 0.5*	3.0 ± 0.0‡	0.5 ± 0.6*
Porcine AF	1.2 ± 0.4†	0 ± 0*	1.8 ± 0.6‡	1.3 ± 0.7†	2.8 ± 0.4‡	0 ± 0*
Porcine NP	2.3 ± 0.9‡	0.5 ± 0.8*	1.7 ± 0.5‡	1.7 ± 0.5‡	2.6 ± 0.5‡	0 ± 0*

*No to low incidence of staining (0–0.5); †low to moderate incidence of staining (0.5–1.5); ‡moderate to high incidence of staining (≥1.5).

Table 2 Mean incidence of staining for alpha and beta subunits in human disc and human articular cartilage

	α_1	α_2	α_3	α_4	α_5	α_6	α_V	β_1	β_2	β_3	β_4	β_5	β_6
Human disc	++	–	–	–	++	–	++	++	–	++	+	+++	++
Articular cartilage*	+++	–	–	–	++	–	+++	++	–	–	–	++†	–

No staining (–), low incidence of staining (+), moderate incidence of staining (++), high incidence of staining (+++).
*Data taken from Ostergaard et al. (1998); high Incidence of staining here represents those sections with a median score of 3–4 in Ostergaard et al.
†Data for $\alpha_V\beta_5$.

Results

Mean scores and standard deviations for each integrin subunit for each tissue are given in Table 1. The most highly expressed integrins in human tissue sections (moderate to high incidence of staining) were α_5 and α_V (Table 1a), and β_1, β_3, β_5 and β_6 subunits (Table 1b). A consistent incidence of staining was also observed for α_1, β_1, β_3 and β_6 subunits (low to moderate staining). A similar staining profile was observed in porcine AF tissue sections, except for the absence of α_V and β_6 subunits in the porcine tissue, and a higher incidence of staining for α_1, α_6 and β_4 subunits. The expression of subunits differed for the porcine NP tissue as compared with either human or porcine AF tissues, with a higher incidence of α_6, β_1 and β_4 subunits. Notably, there was a moderate to high incidence of staining for the α_6 subunit in the NP tissue, expressed at low levels or completely absent in human or porcine AF tissues (Table 1a).

Discussion

This study presents the first available data for the integrin expression profile of cells of the intervertebral disc. Although the data presented here are obtained from a small number of human discs (n = 3), tissue was examined from multiple regions (n = 5) and sections (n = 6). In addition, there was evidence of little or no variability in integrin expression among the tissue sections. Results for the porcine tissue samples, for which we had a larger sample number, showed no more or less variable findings. Because of this low variability and the descriptive nature of this study, data were deemed sufficient for the analyses presented.

The integrin expression profile of human disc tissues had some similarities to that documented for cartilage from normal human femoral heads (Ostergaard et al. 1998; Table 2). Similarities in integrin subunit expression between these two tissues may relate to similarities in the composition of their extracellular matrices. For example, subunits for the dimer $\alpha_1\beta_1$ are expressed by both tissues, which is most commonly recognized as the type II collagen receptor for chondrocytes; however, $\alpha_1\beta_1$ can also mediate adhesion to type VI collagen and to matrilin-1. Both tissues also express subunits for $\alpha_5\beta_1$, which is the primary fibronectin receptor used by chondrocytes; however, α_V, which has been shown to be present in human articular cartilage, was also expressed by human AF and may mediate interactions with fibronectin. Both tissues lacked expression of the

α_2 subunit *in situ*; however, it has recently been reported by that α_2 is expressed by isolated articular chondrocytes and may play a role in adhesion to type II or VI collagen (Forsyth et al. 2002). Whereas chondrocytes have been shown to express α_2, this expression has not been shown at the tissue level, and was also not detectable in human disc tissue in this study.

Some differences are apparent between the human AF tissues studied here and the results for articular cartilage reported previously. Human AF tissue exhibited a higher incidence of staining for the β_3, β_4 and β_6 subunits than human articular cartilage and a slightly lower incidence of staining for the α_v subunit than articular cartilage.

Importantly, porcine AF tissue showed a similar staining profile to human AF tissues, with only a few notable exceptions. Incidences of staining for α_5, β_1, β_3 and β_5 subunits were similar for both human and porcine AF tissues; however, human tissue expressed α_1 and β_4 at lower levels than porcine AF tissue. Notably, the human AF tissue stained for α_v and β_6 subunits whereas the porcine AF did not, and the porcine AF tissue stained for the α_6 subunit whereas the human tissue did not. Neither tissue expressed α_2, α_3, α_4, α_{IIb} or β_2. Similarities between the human surgical specimens and porcine AF tissues were surprising owing to the significant differences in age between these specimens. Similarities in occurrence of the integrin subunits between human and porcine tissues do not imply similarities in function, however, as both integrin specificity and intensity have been found to differ with age and degeneration in human articular cartilage (Loeser et al. 1995). Nevertheless, these similarities reflect the origin of the human specimens from the fibrocartilaginous region of the intervertebral disc.

Differences in integrin expression profiles were also observed between AF and NP tissues in the skeletally immature disc tissues of the pig. Of particular interest is the consistent and moderate to high incidence of staining for the α_6, β_1 and β_4 subunits in NP tissues, as compared with a low incidence of staining for α_6, β_1 and β_4 subunits in the AF. The $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins are classical laminin receptors, of which $\alpha_6\beta_1$ is present in cartilage and may play a role in mediating chondrocyte interactions with laminin (Durr et al. 1996). The α_6 subunit exists in two isoforms, α_{6a} and α_{6b}, and expression of both isoforms is required for mesenchymal cells to differentiate down the chondrogenic pathway, and express types II and X collagen (Segat et al. 2002). Thus

it is possible that NP tissues from young porcine spines may be undergoing chondrogenesis, with the possibility that the expression of α_6 may change with maturity. It is also noteworthy that NP cells may be notochordal and thus derived from the laminin-rich ectoderm where expression of α_6 would be expected. Nevertheless, because of the dramatic differences in staining for this subunit in immature disc tissue, expression of the α_6 integrin may have an important role in contributing to differences seen in the biological responses to environmental stimuli between the NP and AF.

Although this study presents a profile of integrin subunit expression in tissues of the intervertebral disc, neither the functional state of the integrins nor their formation of heterodimers was studied. Both α and β subunits contribute to ligand specificity, so that substituting for either subunit, along with effects of alternative splicing, will affect the nature of the interaction between the integrin heterodimer and its ligand. For this reason, together with the fact that integrins can bind to more than one ligand and that ligands can recognize more than one integrin, the diversity and complexity of integrin–ligand interactions and downstream signalling requires knowledge far beyond the expression of individual integrin subunits (Hynes, 1992). Once functionality is confirmed, additional studies will be important for assessing how functional integrin expression contributes to zonal variations in the interverterbal disc cell response to environmental stimuli.

Acknowledgements

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