Regional regenerative potential of meniscal cartilage exposed to recombinant insulin-like growth factor-I *in vitro*

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It is well recognised that meniscal tears situated within the inner, avascular region do not heal. We investigated the potential effect of insulin-like growth factor-I (IGF-I) in promoting regeneration of meniscal tissue in the inner, middle and outer zones of the meniscus. Sheep menisci were harvested and monolayer cell cultures prepared. Various concentrations of IGF-I were used in the presence or absence of 10% fetal calf serum (FCS). We measured the uptake of radioactive thymidine, sulphur, and proline to assess cell proliferation and formation of extracellular matrix (ECM). IGF-I, in the presence or absence of FCS, increased the formation of DNA and ECM in all meniscal zones. However, the response of the cells from the avascular zone was greater than that from the vascular zone. Our findings indicate that fibrochondrocytes cultured from avascular meniscal tissue have the ability to regenerate when exposed to anabolic cytokines such as IGF-I.

The meniscus is an integral component of the knee and makes a major contribution to its biomechanics. It protects the articular cartilage both by redistributing joint loads and through shock absorption.¹⁻⁴ Meniscectomy alters the normal biomechanics of the knee and accelerates the development of osteoarthritis in most patients.⁵⁻¹⁰

Tears are by far the commonest disorder affecting the meniscus especially in young and middle-aged active adults.^{10,11} Their management varies according to many factors including the size and the site of the tear.^{12,13} The work of King¹⁴ in 1936 and, more recently, that undertaken by other researchers,¹⁵⁻¹⁷ has shown that meniscal healing depends mainly on the vascularity of the zone which has been injured. The peripheral zone has good healing potential, whereas tears in the avascular zone rarely heal.

Several growth factors are involved in the repair and regeneration of musculoskeletal tissues.¹⁸⁻²⁰ Insulin-like growth factor-I (IGF-I) is a major mediator in all stages of wound healing, including inflammation, and its absence dramatically impairs healing in most connective tissues.²¹⁻²³

Our aim was to assess the regenerative potential of meniscal cells (fibrochondrocytes) harvested from the inner, middle, and outer regions of the meniscus when exposed to IGF-I in a dose-dependent manner. In particular, we wished to investigate whether or not

cells from the avascular regions of the meniscus had the ability to proliferate and to produce extracellular matrix (ECM), in a manner similar to those of the vascular region, when exposed to IGF-I *in vitro*.

Materials and Methods

We obtained 12 lateral menisci from six sheep from the local abattoir. The animals were of mixed sex and aged between six and 12 months. After removing the highly vascular anterior and posterior horns, and the peripheral capsular attachments, the menisci were divided radially into slices 5 mm in size. Each radial slice was then divided into three equally spaced zones 3 mm thick: an inner (white-white zone), middle (red-white zone), and outer (red-red zone). All the pieces were grouped according to their zone and digested in 0.02% collagenase (GIBCO; Invitrogen Ltd, Paisley, UK) for 14 hours at 37°C to release the cells from the surrounding ECM. The digests were filtered to remove any undigested material using cell strainers with a pore size of 70 µm (Falcon BD; Fred Baker Scientific, Cheshire, UK). The filtrates were then centrifuged at 1300 rpm for ten minutes to separate the cells. The pellets of cells were re-suspended and cultured in 75 cm² flasks (Greiner Bio-one Ltd, Gloucestershire, UK) containing Dulbecco's Modified Eagle's medium (DMEM; GIBCO, Invitrogen Ltd) supplemented with 10% fetal calf serum (FCS; GlobePharm Ltd, Surrey,

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Fig. 1

Photomicrographs of phase-contrast images of confluent monolayer primary cell cultures of fibrochondrocytes obtained from the inner zone of the sheep meniscus a) at x11 and b) at x72 magnification showing the rounded and spindle-shaped cell types.

UK), 0.1% penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively; GIBCO, Invitrogen Ltd) and 50 µg/ml of ascorbic acid (Sigma-Aldrich Ltd, Poole, UK). The cultures were kept in a CO₂ incubator (5% CO₂ and 95% air) at 37°C.

Once confluent, the cells were trypsinised and randomly divided into two different groups, one containing medium supplemented with 10% FCS and the other containing serum-free medium. They were placed in 96-well plates (Greiner Bio-one) at a cell density of 2×10^4 . Recombinant human IGF-I (R&D Systems Inc, Oxon, UK) was used at concentrations of 0 (control), 1, 10, 100 and 200 ng/ml, and the experiments were performed in triplicate. The cells were exposed to IGF-I for 48 hours in total.

Assessment of cell proliferation. This was measured by the incorporation of radiolabelled thymidine (³H-thymidine; Amersham Biosciences Ltd, Bucks, UK) into newly-formed DNA. In the proliferation experiments, 5 µCi/ml of ³H-thymidine were added to the medium along with the IGF-I. The experiments were carried out in triplicate and the results were compared with control samples.

Assessment of the formation of ECM. This was made by measuring the incorporation of radioactive sulphur (³⁵SO₄; Amersham Biosciences Ltd) into newly-formed glycosaminoglycans (GAGs), and the incorporation of radio-labelled proline (¹⁴C-proline; Amersham Biosciences Ltd), into newly-formed proteins. We added together 5 µCi/ml of ³⁵SO₄ and 0.5 µCi/ml of ¹⁴C-proline along with specific concentrations of IGF-I according to the experimental design. The experiments were carried out in triplicate and the results were compared with control samples.

Scintillation counting. After 48 hours of exposure to IGF-I and the radioactive precursors, the culture medium was removed and the cells washed three times with phosphate-buffered saline (PBS; GIBCO, Invitrogen Ltd) to remove unincorporated radioactive precursors. The fibrochondrocytes present in each well were lysed using 0.2% lysis buffer, and a sample of this digestate was added to 2 ml of scintillation fluid and the incorporation of the radioactive

Fig. 2

The geometric mean (with 95% CI) uptake of ³H-thymidine incorporated into newly-formed DNA in the cells of the inner, middle and outer zones of the meniscus in serum-supplemented medium.

Fig. 3

The geometric mean (with 95% CI) uptake of ³⁵SO₄ incorporated into newly-formed GAGs of the ECM of the inner, middle and outer zones of the meniscus in serum-supplemented medium.

Table I. The geometric means uptake of precursor ($\pm$ 95% CI) when exposed to various concentrations of IGF-I in serum-supplemented experiments

IGF-I (ng/ml)	Thymidine			Sulphur			Proline		
	In	Mid	Out	In	Mid	Out	In	Mid	Out
0	4082 (713)	4002 (862)	5423 (1118)	35 (9)	56 (20)	54 (2)	50 837 (10169)	54 593 (12602)	73 220 (14021)
1	6342 (2225)	7829 (1353)	9531 (2937)	54 (13)	65 (9)	126 (58)	44 126 (3500)	10 787 (1348)	26 424 (20816)
10	5684 (3581)	7772 (1252)	9135 (1109)	85 (7)	84 (14)	95 (53)	111 825 (16750)	49 979 (5943)	51 095 (43156)
100	39 869 (9165)	36 118 (13247)	7922 (2961)	119 (13)	129 (10)	115 (17)	134 981 (5861)	159 100 (11461)	161 272 (18629)
200	47 734 (7236)	38 530 (6885)	16 386 (6786)	140 (47)	236 (32)	162 (30)	186 208 (3407)	167 454 (4505)	175 675 (23587)

precursor(s) measured (disintegrations per minute (DPM) using a scintillation counter (Wallac 1409; PerkinElmer Life Sciences Ltd, Cambridge, UK). Each sample was read for five minutes.

Statistical analysis. This was performed using SigmaStat for Windows computer software (version 2.03, SPSS UK Ltd, Surrey, UK) under the supervision of our Medical Statistics Department. Statistical significance was assessed using two-way analysis of variance taking into consideration both the effect of the concentration of IGF-I used and the zonal origin of the cells. The results for each group, serum-free and serum-supplemented, were analysed separately. When data were not normally distributed, logarithmic transformation of the data was carried out. Tukey's test for multiple comparisons was performed. We considered a p value of < 0.05 to be statistically significant.

Results

In the primary monolayer cell culture, the cells reached confluence in seven to ten days. They were a mixture of phenotypes; oval or spindle-shaped cells which normally exist in the superficial layer of the meniscus, and round-shaped cells which are found predominantly in the deep layer (Fig. 1).

In the serum-supplemented media group, there was an eight- to ten-fold increase in the uptake of thymidine in the inner and middle zones using concentrations of IGF-I of 100 or 200 ng/ml, compared with the control experiments ($p < 0.001$). In the outer zone there was a 3.5-fold increase in the uptake of thymidine at a concentration of IGF-I of 200 ng/ml ($p = 0.002$) (Fig. 2). The uptake of sulphur increased by between three- and five-fold in all three zones of the meniscus when exposed to a concentration of IGF-I of 200 ng/ml ($p < 0.05$) (Fig. 3). Similarly, the uptake of radiolabelled proline was increased three-fold in all three zones at concentrations of IGF-I 100 and of 200 ng/ml ($p < 0.001$; Fig. 4; Table I).

In the serum-free media group, there was a four- to five-fold increase in the uptake of thymidine in all zones of the meniscus ($p < 0.05$) (Fig. 5). The uptake of sulphur was increased by 2.5-fold in all three zones of the meniscus using concentrations of IGF-I of 100 and 200 ng/ml ($p < 0.05$; Fig. 6). The uptake of proline was increased by ten- to 16-fold in all three meniscal zones using concentrations of IGF-I of 10, 100, and 200 ng/ml ($p < 0.05$; Fig. 7; Table II).

Overall, in every experiment, there was a statistically significant interaction between the concentration of IGF-I and

the meniscal zone ($p < 0.05$). In addition in both the serum-free and in the serum-supplemented experiments, irrespective of the precursor under investigation, the differences in the mean value of uptake of precursors were statistically

Fig. 4

The geometric means (with 95% CI) uptake of ^{14}C -proline incorporated into newly-formed proteins of the ECM of the inner, middle and outer zones of the meniscus in serum-supplemented medium.

Fig. 5

The geometric mean (with 95% CI) uptake of ^3H -thymidine incorporated into newly-formed DNA in the cells of the inner, middle and outer zones of the meniscus in serum-free medium.

Fig. 6

The geometric mean (with 95% CI) uptake of $^{35}\text{SO}_4$ incorporated into newly-formed GAGs of the ECM of the inner, middle and outer zones of the meniscus in serum-free medium.

Fig. 7

The geometric means (with 95% CI) uptake of ^{14}C -proline incorporated into newly-formed proteins of the ECM of the inner, middle and outer zones of the meniscus in serum-free medium.

Table II. The geometric means uptake of precursor ($\pm$ 95% CI) when exposed to various concentrations of IGF-I serum-free experiments

IGF-I (ng/ml)	Thymidine			Sulphur			Proline		
	In	Mid	Out	In	Mid	Out	In	Mid	Out
0	13 943 (637)	21 582 (622)	32 860 (3757)	133 (9)	158 (13)	183 (40)	5617 (508)	5300 (959)	13 002 (4737)
1	83 031 (9183)	64 974 (4673)	125 209 (6593)	141 (10)	138 (6)	183 (7)	10 439 (8895)	9619 (377)	10 988 (1623)
10	82 495 (3106)	64 957 (1278)	112 625 (7217)	203 (11)	180 (12)	276 (21)	51 733 (3510)	61 808 (14174)	71 512 (3764)
100	65 262 (6237)	64 210 (1741)	99 186 (5758)	345 (34)	326 (17)	437 (97)	85 259 (6867)	107 901 (4585)	922 253 (3804)
200	85 633 (5528)	82 258 (2508)	116 221 (4206)	380 (31)	349 (5)	475 (25)	153 886 (17740)	177 915 (7293)	159 125 (14923)

significant ($p < 0.05$) at all concentrations of IGF-I and in all meniscal zones.

Discussion

The sheep meniscus has been shown to have peripheral vascular and inner avascular zones which respond to injuries like those of the human meniscus.²⁴⁻²⁶ In addition, Joshi et al²⁷ reported that the sheep meniscus is an excellent experimental model since its mechanical properties closely match those of the human meniscus.

Studies have shown that 'red-red' tears, which involve the outer meniscus, can heal after surgical repair whereas 'white-white' tears, involving the inner avascular zone, do not.^{28,29} It has been postulated that the lack of certain cytokines or growth factors may account for this variation. Arnoczky, Warren and Spivak³⁰ in their experiments on meniscal tears involving the avascular zone in dogs, showed that the introduction of a fibrin clot around a meniscal tear enhanced meniscal regeneration. Ochi et al³¹ reported that rasping the edge of meniscal tears created in the avascular zone of rabbit menisci increased the production of cytokines which presumably was responsible for improving healing.

We chose IGF-I because of its known stimulatory effect on musculoskeletal soft tissues. Several studies have shown that IGF-I contributes to the healing of tendons^{18,32,33} and

repair of articular cartilage.³⁴⁻³⁷ However, there have been no studies in the literature which have explored the role of IGF-I in meniscal regeneration, although a few have investigated the effects of growth factors such as platelet-derived growth factor (PDGF), fibroblast-derived growth factor, and transforming growth factor on meniscal tissue.^{24,38-41} Spindler et al²⁴ reported that unlike cells from the vascular zone, fibrochondrocytes cultured from the inner avascular zone were not stimulated by PDGF. They concluded that the inner zone lacks the ability to regenerate.²⁴

In our study, IGF-I was clearly capable of stimulating the activity of fibrochondrocytes in keeping with cell proliferation and formation of ECM in all zones of the meniscus including the avascular zone. In addition, IGF-I stimulated the activity of fibrochondrocytes when used either singly or in combination with 10% FCS.

Interestingly, IGF-I increased the uptake of thymidine and presumably the formation of DNA more in the inner zone than in the outer zone of the meniscus in the presence of serum.

Our findings indicate that meniscal cells, and more importantly cells from the avascular zone, are capable of responding favourably to the addition of IGF-I. After stimulation with this growth factor, these cells express their intrinsic potential to proliferate and generate new ECM. Finally, our results suggest that it may be possible in the

future to augment meniscal repair and to advance tissue-engineering methods in order ultimately to create meniscal replacements by using suitable growth factors.

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