NEOVASCULARISATION OF THE MENISCUS WITH ANGIOGENIN

AN EXPERIMENTAL STUDY IN RABBITS

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Angiogenin, a potent blood vessel inducing protein, was implanted into experimentally injured menisci of 75 New Zealand white rabbits. Localised neovascularisation occurred in 52% of the angiogenin-treated animals, and in 9% of the controls.

Neovascularisation induced by angiogenin may enhance healing of injuries within the poorly vascularised meniscal fibrocartilage, and improve the results of meniscal repair.

The blood supply to the menisci of the knee is sparse and they heal poorly after injury. In a now classic experiment in dogs, King (1936) showed that injuries in the vascularised periphery healed after repair, but those in the avascular body of the meniscus did not. This work has been confirmed in dogs, pigs, sheep, and rabbits (Heatley 1980; Cabaud, Rodkey and Fitzwater 1981; Arnoczky and Warren 1983; Ghadially, Wedge and Lalonde 1986). All these studies linked meniscal healing to blood supply. Arnoczky and Warren (1982) demonstrated (by means of a micro-injection technique) that the blood supply of the human meniscus is confined to its periphery.

Because meniscal tears in the avascular body fail to heal, partial or complete excision has been the standard treatment for symptomatic meniscal injuries. However, because the meniscus contributes to load bearing (Walker and Erkman 1975), and enhances knee stability (Levy, Torzilli and Warren 1982) meniscal excision has deleterious consequences for the knee and repair has been advocated (Wirth 1981; Scott, Jolly and Henning 1986).

The blood supply is sufficient for predictable healing only if the tear is in the periphery thus efforts at repair have focused on injuries to the meniscal rim (Hamberg, Gillquist and Lysholm 1983; DeHaven 1985) which comprise only a small proportion of all tears.

We report for the first time biochemically induced neovascularisation of the meniscus using angiogenin, a

123 amino acid protein that has been recently isolated and purified (Fett et al 1985; Shapiro et al 1987). Its angiogenic potency has been documented in the chick embryo and the rabbit cornea (Fett et al 1985; Strydom et al 1985) where neovascularisation occurs in response to very small amounts of the protein.

MATERIALS AND METHODS

Angiogenin. Angiogenin, derived from human plasma, isolated and purified to homogeneity, was suspended in an aqueous solution of 1% methyl cellulose. Details of the preparation technique have been described by Shapiro et al (1987).

Methyl cellulose (4000 Cp) was autoclaved and then dissolved (1% weight/volume) in sterile water by stirring overnight at 4°C. Lyophilised, salt-free samples of angiogenin were then suspended in the solution by gentle stirring for two hours at 4°C. Small quantities of the suspension (10 µm) were placed on a clean, dry, mylar sheet and air-dried under laminar flow conditions to form discs 3 mm in diameter, each containing 100 ng of angiogenin. The sheet was placed in a sterile, square Petri dish, in a desiccator, and lyophilised for one hour to ensure that the discs were completely dry. Methyl cellulose discs were prepared containing no angiogenin for use as controls. Under sterile conditions, each disc was removed from the plastic sheet with forceps, folded and inserted into the sharp end of a 20-gauge cannulated needle.

Discs were implanted into the avascular body of the lateral meniscus of New Zealand white rabbits weighing between 5 and 7 kg. There were 75 angiogenin implants and 22 controls.

Operative technique. The rabbits were anaesthetised with intramuscular acepromazine maleate (1.5 mg per kg body-weight) and ketamine (25 mg per kg), and a local

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subcutaneous injection of 1% xylocaine. The anterior, right lower extremity was shaved, cleaned with 70% alcohol solution, and draped with sterile towels. The knee was exposed through a lateral parapatellar incision. The patella was dislocated medially, the femoral condylar origin of the extensor tendon was divided and the knee was flexed. The collateral and cruciate ligaments were not disturbed. The anterior half of the lateral meniscus was visualised at ten-times magnification with a binocular dissecting microscope. With a small scalpel, a 2.5 by 0.8 mm horizontal pocket was made in the avascular body of the meniscus starting 2 mm from its peripheral margin (Fig. 1). The pocket was just large enough to admit the sharp end of the 20-gauge needle containing the folded methyl cellulose disc. The obturator from a spinal needle was used to eject the disc into the pocket. A 2 mm vertical incision was then made medial to the pocket to simulate a longitudinal laceration (Fig. 1). This incision was only of partial-thickness because full-thickness incisions left an unstable fragment that could displace into the joint and lead to degenerative changes within the meniscus.

Fig. 1

Diagram of the implantation pocket and the incision in a rabbit meniscus.

The knee was extended, the patella was reduced and the tissues were irrigated with 0.9% saline solution containing gentamicin 500 mg per litre. The retinaculum was closed with interrupted figure-of-eight sutures of 2-0 absorbable vicryl and the skin was closed with a running subcuticular suture of 4-0 vicryl. The rabbits were permitted to run freely in their cages and were fed a standard diet.

Harvesting samples. Groups of rabbits were killed at intervals of three, six, eight, nine, 12 and 26 weeks using a lethal intravenous injection of acepromazine maleate (3 mg per kg) and ketamine (50 mg per kg). The knees were examined using the dissecting microscope, the

observer being unaware of the identity of the sample implanted.

Grading system. The menisci were graded as follows:

Grade 0 denoted absence of neovascularisation; the meniscus was not inflamed and there was no healing around or in the pocket or medial incision;

Grade I signified the presence of new blood vessels growing over the anterior horn of the meniscus; the vessels were contained within a pannus of connective tissue covered by synovium (Fig. 2).

Histology. The meniscus was fixed in 10% neutral buffered formalin, decalcified, embedded in paraffin, sectioned at 6 μ m perpendicular to the coronary ligament and examined histologically using standard haematoxylin and eosin staining techniques. Blood vessels containing red corpuscles were so readily seen under the light microscope that verification of their presence was unnecessary.

RESULTS

Neovascularisation (grade I) was observed in 39 of the 75 menisci treated with angiogenin (52%). In all cases of neovascularisation, the response was localised to the area of sample implantation.

In 20 of the 22 controls the menisci were grade 0 (91%), and the remaining two were grade I. The grade 0 knees were devoid of any neovascularisation, synovial proliferation or meniscal healing. The pocket and the medial incision were clearly visible and easily probed. Healing of the medial incision was not observed in any of these specimens.

The difference between the angiogenin and the control group was significant ($p < 0.0001$ by the Bernoulli trial method).

Timing of neovascularisation. In the angiogenin group, neovascularisation was seen in three of eight animals killed before four weeks, in 30 of 53 killed between four

Fig. 2

Grade I meniscus: prominent new blood vessels lie atop the meniscus ($\times 12$).

and ten weeks and in six of 14 killed after more than ten weeks.

In the control group, the two grade I responses were seen at six and eight weeks.

Histology. In grade 0 the body of the meniscus was avascular (Fig. 3). There was a little necrosis around the surgical incision but no signs of inflammation, neovascularisation, repair, healing, fibrosis or cell division. The appearances were the same as in the normal meniscus.

The grade I responses were characterised by vascular connective tissue invading the meniscus from both the dorsal and the ventral surfaces with a thin covering layer of synovial cells. The vessels were arterioles, venules, and capillaries, 8 to 50 μ m in diameter. The vessels penetrated the body of the meniscus and fibroblasts had invaded the area of surgical injury (Fig. 4). Acute inflammatory cells were few. The synovial tissue covering the vascular connective tissue and the meniscal surface was only two cell layers thick and did not contain lymphocytic follicles. The synovium did not invade the meniscal tissue.

DISCUSSION

Without a blood supply, the knee meniscus heals poorly, if at all, following injury. Haematoma does not form; fibrin clot, the cellular precursors and the natural scaffolding for structuring a scar are all absent. The meniscal tear remains vulnerable to shear and compressive forces as the knee flexes and extends. A combination of meniscal stabilisation using a suture technique and biochemically induced neovascularisation may offer the potential of repairing and healing tears in the avascular body of the meniscus. This study shows that angiogenin can induce angiogenesis in the meniscus.

Is a blood supply really necessary for meniscal healing? In an attempt to answer this question, investi-

gators have examined local cellular elements including the synovium and fibrochondrocytes to detect whether or not they can muster a reparative response (Potenza and Herte 1982; Webber, Harris and Hough 1985; Kleiner et al 1986). In normal tissue repair, bleeding provides fibrinous structure, cellular precursors, and mitogenic and chemotactic factors that may play a role in stimulating reparative cells (Peacock 1984; Webber et al 1985). In the absence of bleeding, different mechanisms of repair may be at work. Webber et al (1985) showed that fibrochondrocytes in cell culture are capable of replication and matrix production in response to pituitary fibroblast growth factor and human platelet lysate.

Heatley (1980) found chondrocytes clustered near the margin of an incision in the rabbit meniscus, and that healing of a small incision ensued if the injury extended into the synovial margin. However, the healing potential of larger incisions and of those remote from the synovial periphery was very limited. The invasion of synovial cells was thought to precede healing, and the relative avascularity of the reparative tissue was noted. Veth et al (1983) reported fibrous healing of longitudinal incisions in the avascular portion of the rabbit meniscus without the benefit of a blood supply. They considered that vascularity might be unnecessary as long as a source of reparative cells was at hand.

We could not, however, confirm this conjecture from our data. In our study, avascular healing of longitudinal tears was absent in all 97 knees. Healing occurred only if vascular tissue invaded the area of surgical injury. This finding was not unexpected since the surgical incisions in this experiment did not extend to the periphery where a source of reparative cells or blood vessels might be found.

Vascular tissue can be brought in surgically. Ghadiali et al (1986) sutured a flap of synovium to the meniscal

Fig. 3

Fig. 4

Figure 3 – Photomicrograph of Grade 0 meniscus. No new blood vessels or fibrovascular healing is seen (haematoxylin and eosin $\times$ 100). Figure 4 – Photomicrograph of the meniscus shown in Figure 2. New blood vessels are visible within the meniscal body. Neovascularisation extends into the meniscal parenchyma beyond the synovial pannus. The overlying synovium is only a few cell layers thick and few inflammatory cells are seen (haematoxylin and eosin $\times$ 100).

tear and induced healing. Arnoczky and Warren (1983) drilled holes from the periphery of the meniscus into the avascular body to permit the ingress of reparative cells and promote healing. Using this technique, Zhongnan et al (1988) demonstrated fibrovascular healing in dog menisci. Recently, Arnoczky, Warren and Spivak (1988) placed a fibrin clot directly into 2 mm punctures in dog menisci and showed that the holes filled with an avascular tissue. Thus, without blood vessels to deliver fibrin clot, the direct surgical implantation of a clot may substitute for vascularity.

Angiogenin was first isolated from the culture medium of a human colon adenocarcinoma cell line and was thought to be a tumour-specific protein. However, it has since been detected in normal human cells and has been purified from normal human plasma (Shapiro et al 1987). It is a single chain, non-glycosylated polypeptide of 123 amino acids with a high iso-electric point (> 9.5) (Strydom et al 1985). It is angiogenic *in vivo* in both the rabbit cornea and the chorio-allantoic membrane of the chick embryo, where it is active with doses as low as 1 ng (Fett et al 1985). It differs from other known angiogenic agents in that it is not an endothelial cell mitogen, has no unusual affinity for heparin, and, in particular, was isolated using an assay designed specifically to detect angiogenesis. For this study, a dose of 100 ng was chosen because this amount had been consistently effective in previous work in the rabbit cornea, and because pilot studies in the knee demonstrated no advantage from using larger or smaller doses from 10 to 1000 ng. The diffusion distance between the implanted sample and the nearest source of blood vessels in our meniscal model was

about 2 mm, similar to that found to be effective in the rabbit cornea.

There may be advantages in the use of biochemical angiogenesis within avascular meniscal fibrocartilage. The new blood vessels and connective tissue provide a ready source of cellular, mitogenic and chemotactic reparative factors. As vascularity is part of the normal wound healing response, angiogenesis may provide a more sustained stimulus to stable healing especially in more extensive injuries. In our study, the histological picture resulting from the application of angiogenin was that of the ingrowth of benign vascular fibrous tissue. Fibrochondrocyte necrosis was absent, inflammatory cells were rarely seen and the synovial cell lining did not hypertrophy. The six to eight week time course for neovascularisation is similar to that found by Zhongnan et al (1988) in meniscal trephination suggesting that angiogenin promotes healing but does not hasten its time course.

Summary. Angiogenin induces neovascularisation in the avascular meniscal body. The localised response is brisk enough to induce vigorous vascular fibrous tissue ingrowth. Biochemically induced neovascularisation may prove useful to enhance healing of meniscal injuries.

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