AP

Influence of Actin Cytoskeleton on Intra-articular and Interstitial Fluid Pressures in Synovial Joints

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Fibroblast microfilamentous actin (F-actin) influences interstitial fluid pressure via linkages to collagen in rat skin (Berg *et al.,* 2001). The present aims were to determine whether the actin cytoskeleton of synovial endothelium, fibroblasts, and synoviocytes influences *in vivo* (i) fluid exchange between a joint cavity and synovial microcirculation and (ii) extracellular fluid pressures in joints. Rabbit knee joints were treated intra-articularly with the F-actin disrupting drugs cytochalasin D and latrunculin B while joint fluid pressure P_j was recorded. In joints injected with small volumes of control solution, P_j fell with time (-0.05 ± 0.01 cm H_2O min^{-1}, mean $\pm$ SEM, $n = 9$, equivalent drainage rate 3.9 μl min^{-1}). Cytochalasin or latrunculin reversed this in ~4 min *in vivo*; P_j increased with time, e.g., $+0.12 \pm 0.04$ cm H_2O min^{-1} at 200 μM cytochalasin (equivalent filtration rate into joint 6.6–12.5 μl min^{-1}, $n = 4$), with a cytochalasin EC$_{50}$ of 45 μM. Plasma γ-globulin clearance into the joint cavity was also increased. *Post mortem,* cytochalasin did not reverse dP_j/dt and had no more effect on P_j than did control solution. Also, when synovial interstitial fluid pressures were measured by servonull micropipette *post mortem* (control -0.95 ± 0.37 cmH_2O, $n = 18$) cytochalasin had no significant effect on interstitial pressure over 60 min, even at 1 mM. It was concluded that synovial endothelial F-actin has an important role in the normal synovial microvascular resistance to fluid filtration and plasma γ-globulin permeation and is thus a potential link between pro-inflammatory mediators and arthritic joint effusions. The results provided no support for the hypothesis that synoviocyte F-actin influences the swelling tendency of synovial matrix and hence extracellular fluid pressures, in contrast to the findings of Berg *et al.* (2001) in rat dermis. © 2001 Academic Press

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INTRODUCTION

Synovium is a thin, mesenchyme-derived sheet of tissue responsible for the generation, encapsulation, and drainage of synovial fluid in joints. A superficial network of fenestrated microvessels filters fluid slowly into the joint cavity when intra-articular pressure is low, as in extended joints. In synovitis a breakdown of the endothelial barrier leads to the development of a protein-rich effusion in the joint cavity (Wallis *et al.,* 1987). When synovial fluid pressure is raised, e.g., by flexion, the net trans-synovial flow reverses direction and fluid is driven out of the joint cavity. The fluid passes between the discontinuous synoviocytes (specialized fibroblasts; Edwards, 1999) that line the surface and drains into a subsynovial lymphatic system. The intersynoviocyte pathway comprises a matrix of types I, III, IV, V, and VI colla-

gen, hyaluronan, sulfated proteoglycans, and fibronectin (Levick *et al.,* 1996), and its level of hydration is an important factor influencing its permeability.

Our interest in the role of filamentous actin (F-actin) in synovial cells arose from reports that F-actin in fibroblasts modulates the interstitial fluid pressure and hydration of rat skin (Reed *et al.,* 1997; Berg *et al.,* 2001). F-actin is a ubiquitous cytoskeletal microfilament that is present in endothelium (see below), fibroblasts (Ribeiro *et al.,* 1997), and their close relatives, the synoviocytes that line a joint cavity (Ward *et al.,* 1999). The F-actin is linked to the extracellular matrix through membrane integrins (Takenaga *et al.,* 1990). In endothelium, the actin microfilaments also link with the intercellular junction protein complex (Dejana *et al.,* 1995). The work of Reed's group, using servonull micropipettes to measure pressure, indicated that the fibroblast cytoskeleton exerts a tonic tension on interstitial collagen through $\alpha_2\beta_1$-integrins, thereby opposing the interstitial glycosaminoglycan swelling pressure and the innate tendency of the tissue to hydrate. Disruption of this equilibrium, using antibodies against $\alpha_2\beta_1$-integrin or the F-actin disrupting agent cytochalasin, caused interstitial fluid pressure to fall to more subatmospheric values over 30–60 min, due to expression of the unopposed gel swelling pressure. In synoviocytes the cytoskeleton is likewise linked to the extracellular matrix by β_1 integrins (Rinaldi *et al.,* 1997) and to hyaluronan through CD44 (Lacy and Underhill, 1985; Henderson *et al.,* 1993; Asari *et al.,* 1995). One aim, therefore, was to investigate whether synoviocyte F-actin influences extracellular fluid pressures in joints.

The negative pressure responses described by Reed's group were clearly demonstrated only *post mortem,* when circulatory stasis prevents a secondary rise in interstitial hydration. *In vivo,* by contrast, cytochalasin caused increases in the hydration and the albumin content of rat dermis, indicating that F-actin also contributes to endothelial barrier function in rat skin (Berg *et al.,* 2001). This is in keeping with studies of continuous endothelium monolayers *in vitro,* which show that cytochalasin disrupts phalloidin-labeled microfilaments and causes cell retraction, intercellular gap formation, and a raised permeability (Shasby *et al.,* 1982; Alexander *et al.,* 1988; Haselton *et al.,* 1989; Holda and Blatter, 1997; Kevil *et al.,* 1998). Moreover, changes in F-actin distribution and disruption of the peripheral F-actin ring are features of inflammation in venular endothelium (Baldwin and Thurston, 1995; Ehringer *et al.,* 1999). *In vivo,* intravascular cytochalasin causes edema, a fall in the albumin reflection coefficient, and a rise in the microvascular filtration capacity (Shasby *et al.,* 1982; Korthuis *et al.,* 1991; Ermert *et al.,* 1995). In view of the above findings, a second aim was to investigate whether F-actin influences the movement of fluid across the fenestrated capillaries of synovium and hence whether it has a potential role in the formation of joint effusions.

The above aims were addressed by experiments using two F-actin disrupting drugs. Cytochalasin D is a fungal metabolite that disrupts actin microfilaments in minutes, primarily by capping the barbed, growing end of the actin filament. Actin filaments are dynamic structures that undergo a continuous, rapid, treadmill-type turnover, in which monomeric G-actin is continuously removed from one end and added to the opposite end at a rate of $>4.5\ \mu\text{m min}^{-1}$ (Symons and Mitchison, 1991; Alberts *et al.,* 1994). Blockage of the growing end by cytochalasin is thus quickly followed by filament disassembly. Rapid disassembly of F-actin has been confirmed both in endothelium (Holda and Blatter, 1997; Wang, 1998) and in fibroblasts (Ribeiro *et al.,* 1997). Additional mechanisms may contribute to F-actin disruption at high drug concentrations (Cooper, 1987).

To confirm the specificity of cytochalasin D-induced effects, we also carried out experiments with latrunculin B, a Red Sea sponge toxin that inhibits F-actin polymerization by a different mechanism. Latrunculin binds to the cytosolic pool of G-actin and thus reduces its availability, which leads to disruption of the rapidly turning over F-actin microfilaments (Spector *et al.,* 1983; Coué *et al.,* 1987; de Oliveira and Mantovani, 1988).

METHODS

In an initial series of experiments *in vivo,* F-actin disrupting drug was injected into the joint cavity of

rabbit knees under anesthesia and resulting changes in joint fluid pressure (P_j) were recorded through a cannula for 30 min. A fall in pressure indicated net drainage of fluid from the cavity, whereas a rise indicated net filtration into the cavity. The experiment was repeated on the contralateral joint using a control solution or low dose of drug. The pressure response in the joint cavity to cytochalasin D was also studied *post mortem*. The reasoning behind the latter study was that any increase in the swelling tendency of synovial interstitium will cause the absorption of some intra-articular fluid *post mortem* and thus be revealed by a fall in articular fluid pressure. In a second series of experiments, designed to reproduce closely the servonull micropipette method of Berg *et al.* (2001), minute boluses of drug at various concentrations were injected directly into the exposed synovial tissue *post mortem,* and interstitial fluid pressure in the adjacent tissue was monitored by a servonull micropipette system for up to 60 min.

Experimental Preparation in Vivo

New Zealand white rabbits weighing 2.5–3 kg were anesthetized with 30 mg kg^{-1} sodium pentobarbitone plus 500 mg kg^{-1} urethane iv and tracheostomized. Anesthesia was maintained at sufficient depth to abolish the corneal blink reflex using intravenous 15 mg sodium pentobarbitone plus 250 mg urethane every 30 min. Procedures conformed to local animal legislation and animals were killed by an overdose of iv sodium pentobarbitone at the end of the experiment. The intra-articular cannulation and pressure-recording systems were as described by Coleman *et al.* (1999).

Intra-articular Injections and Rate of Change of Joint Fluid Pressure, dP_j/dt

A 21-gauge cannula was inserted into the joint cavity and connected to a water-calibrated differential pressure transducer to measure P_j. A bolus of 150–400 μl of test solution was injected into the joint cavity through the pressure line, raising P_j to just above atmospheric pressure, and P_j was recorded for 30 min. Since P_j is related to the volume of fluid V present in the joint cavity through a known compliance curve

(Knight and Levick, 1982, 1983), the rate of change of pressure dP_j/dt served as a measure of $dV/dt,$ i.e., the net trans-synovial flow ($\dot{Q}_s$). Slopes on the chart record were fitted by ruler.

Interstitial Pressure Measurements by the Servonull Micropipette System Post Mortem

The servonull feedback system (Vista Electronics Co., Ramona, CA) utilized changes in electrical resistance in a 1 M NaCl-filled micropipette, caused by minute flows of interstitial fluid through the tip, to control a pump that adjusts the pressure in the micropipette until it balances that of the interstitial fluid. The method has been evaluated by Wiig *et al.* (1981). The system used here was as described by Miserocchi *et al.* (1990). Briefly, glass micropipettes of outer diameter 1 mm with ground, beveled tips of diameter of 2–5 μm were filled with 1 M NaCl, which was colored with lissamine green for tip visualization. The system's P23XL pressure transducer (Gould Instruments, Cleveland, OH) was precalibrated with a water column. The linearity of calibration of each micropipette was checked by inserting it into a sealed chamber of normal saline connected to a second water column. Zero level (atmospheric pressure) was set with the tip touching a small pool of normal saline level with the micropuncture site. Output was recorded on a thermal oscillograph (Gould Instruments).

Interstitial fluid pressures were measured in the synovial lining of joint cavities that were opened immediately after the animal was killed by an anesthetic overdose. A central flap of tissue was raised by means of a transverse incision across the insertion of the patellar tendon, extended proximally by two parallel incisions along the medial and lateral sides of the patellar ligament and patella. The resulting flap was retracted to access the joint cavity. The lateral margin of the joint capsule was then retracted to expose a sheet of lateral suprapatellar synovium. The underside of the retracted tissue was supported by a plasticine mold. The space between the tissue, the microscope objective, and the micropipette was enclosed by a continuous seal of polyvinyl chloride/ethylene film (Cling Film, Woolworths, UK) to maintain local humidity. Superfusion was not used, in order to avoid

overhydration of the interstitium prior to the pressure measurements.

After the initial, control measurements of synovial and subsynovial interstitial pressures (see below), 1.5 μl of test solution was injected as superficially as possible under the exposed lateral suprapatellar synovium, using a 34-gauge needle and a 10-μl Hamilton syringe (Hamilton, Sutton, England), which was held at a shallow angle. Since the needle was thicker than the synovium, the fluid was delivered into the subsynovium rather than the synovium itself. The volume injected was too small to raise a visible surface bleb.

The micropipette was mounted in a MO-102 micromanipulator (Narishige, Tokyo, Japan). After the zero pressure reading was checked with the tip touching the tissue surface, the tip was advanced into the synovium under stereomicroscopic guidance (SMZ-2T stereomicroscope, Nikon, Tokyo, Japan). The vertical depth of the tip was calculated as $h \cdot \sin \theta$, where θ was the angle of insertion ($\sim$45°) and h the distance advanced obliquely, as read from the micromanipulator scale in micrometers. Since the arithmetic mean thickness of rabbit synovium in the suprapatellar pouch is 16–19 μm, and that of the subsynovium is $\sim$100 μm (Knight and Levick, 1983; Levick and McDonald, 1989a), measurements taken at tip depths of <10 μm were classified as synovial interstitial pressures and those at 20–35 μm were classified as subsynovial interstitial pressures. The region between 10 and 20 μm was ignored due to the uncertainty over tissue type near the synovium/subsynovium boundary during a blind penetration. After stable pressures were recorded for 10 s or so in the synovium and subsynovium, the micropipette was withdrawn and the zero rechecked. Readings were accepted if the pipette zero reading was the same before and after insertion, and if the tissue surface was not visibly distorted by entry of the micropipette. Changing the gain of the servonull system without a consequent change in recorded pressure provided a further check on the system in control experiments. Pressure measurements were repeated at 10-min intervals for an hour, moving the micropipette tip around the perimeter of the injection site so that each measurement was 10–15 μm from the previous site but equidistant from the injection center.

Measurements were taken at points 1 mm radially from the center of the injection site. The radial distance was marked lightly on the tissue surface at four points at the outset, using another micropipette dipped in a lissamine green solution. The distance selected for measurements represented a compromise between avoiding the central region where pressure was raised by the injection yet remaining within the region reached by the drug. Following the approach of Berg *et al.* (1998), measurements were made just beyond the edge of the injected bolus, which would be at 0.7-mm radius for a 1.5-μl sphere. A pilot study showed that pressure fell from +7.5 cm H_2O in the center of the injection site to −1.5 cm H_2O at the 1-mm radial distance. Since a small solute such as cytochalasin, with a diffusivity similar to that of glucose, achieves 99% diffusional equilibration across 1 mm in 9.3 min, even when unaided by convection, it was concluded that tissue only $\sim$0.3 mm from the edge of the initial bolus will be exposed to drug within minutes of its injection, albeit at subnominal concentrations.

Small Solute Clearance from Joint Cavity

The intra-articular concentration of a small, rapidly diffusible solute such as cytochalasin D (508 Da) must decay with time *in vivo* due to diffusional clearance into the fenestrated capillaries and periarticular tissue. A preliminary calculation of clearance by the microcirculation predicted a substantial rate of decay, as follows. For flow-limited (i.e., maximal) microvascular clearance by an estimated plasma flow of $\sim$40 μl min^{-1} per knee, the solute half-life would be $\sim$17 min in a joint cavity containing 1 ml fluid (corresponding pressure $\sim$6 cm H_2O). To test this experimentally, the clearance of three small, colored solutes was assessed *in vivo* and *post mortem* by injecting 1 ml of solution into the joint cavity at time zero and aspirating small aliquots at 8, 16, and 32 min. Solute concentrations were assayed by optical absorbance in a spectrophotometer. The three solutes were acridine orange (370 Da, lipid and water soluble, absorption peak 482 nm), patent blue V (582 Da, water soluble only, absorption peak 636 nm), and Evans blue (963 Da, water soluble only, absorption peak 605 nm). Cytochalasin itself had no convenient absorption maximum for assay.

Plasma Globulin Permeation into Joint Cavity

To assess the transcapillary permeation of native plasma protein into the joint cavity, a pair of joints was infused with 100 μM cytochalasin D and control solution, respectively. Fluid was aspirated at 15-min intervals and replaced by fresh solution, and the experiment continued for >1 h. Aspirates, along with a sample of ear vein plasma, were analyzed for native γ-globulin by polyacrylamide gel electrophoresis (NuPage Electrophoresis System, Novex Electrophoresis, Frankfurt, Germany). Gels and standards stained with Coomassie blue were read using a Fluor-S MultiImager system (Bio-Rad, Hercules, CA). Net flux of γ-globulin into the joint cavity was calculated as concentration $\times$ intra-articular volume/time.

Materials

Cytochalasin D and latrunculin B were purchased from Calbiochem (Nottingham, UK). Cytochalasin D (1 mg) was dissolved in 0.5 ml dimethyl sulfoxide, which was then added drop by drop to phosphate-buffered saline (pH 7.4) with continuous rapid stirring to prevent precipitation, until the required dilution was reached. The infused concentrations were 0, 1, 10, 25, 50, 100, and 200 μM cytochalasin D, each of which was studied in triplicate or more. Due to its high cost, only a limited study was carried out with 1 mM cytochalasin, the concentration studied by Berg *et al.* (2001). The broad-spectrum protease inhibitor α_2-macroglobulin was included in one experiment with 200 μM cytochalasin D to test whether activation of tissue protease played any role (Unemori and Werb, 1986). Latrunculin was infused at 100 μM. Control solutions contained the same concentration of dimethyl sulfoxide as the contralateral test solution. The Ringer solution comprised 147 mM Na^+, 4 mM K^+, 2 mM Ca^{2+}, and 156 mM Cl^- at pH 7.2 (Baxter Healthcare Ltd., Thetford, Norfolk, UK).

Statistical Methods

Results were compared using Student's *t* test or analysis of variance (ANOVA) as appropriate, with $P \leq 0.05$ considered a significant difference. EC_{50} was
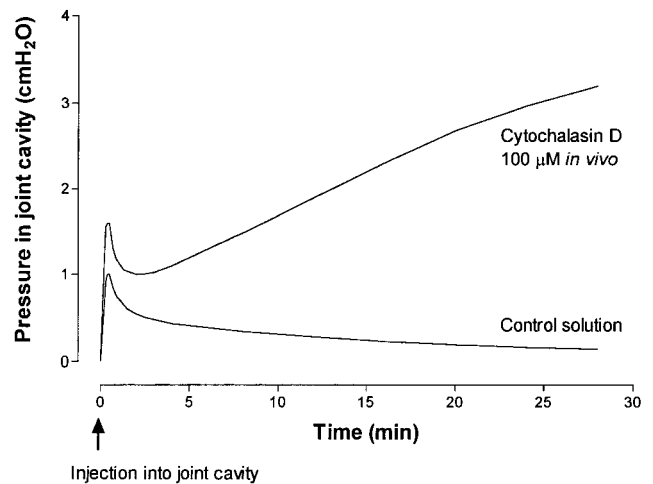

FIG. 1. Effect of intra-articular injection of 100 μM cytochalasin D or vehicle (Ringer solution containing 2.5% dimethyl sulfoxide) on pressure in contralateral knees of the same animal *in vivo*. The initial spike was caused by the injection volume, $\sim$300 μl. In the control joint the pressure then decayed as the injectate drained out through the synovial lining. Cytochalasin halted the decay in 3 min and initiated a rise in intra-articular pressure, indicating the development of an effusion. Measurements of dP_j/dt were taken at the time of maximum rate of increase (t_{max}), $\sim$10 min in this case.

estimated by fitting a 4-parameter logistic equation (top, bottom, Hill slope, EC_{50}; GraphPad Prism, San Diego, CA) to the logarithmic dose–response curve. Means are followed by their standard errors throughout.

RESULTS

Effect of Cytochalasin on Intra-articular Pressure and Microvascular Filtration in Vivo

When control solution was injected into the joint cavity, raising pressure initially to 1–2 cm H_2O, P_j decayed slowly over the next 30 min ($n = 9$) (Fig. 1). The decay is due to drainage of the injected fluid through the synovial interstitial pathway into the subsynovium, where the endogenous fluid pressure is subatmospheric (Scott *et al.,* 2000b). In marked contrast, when cytochalasin D solution at $\geq$50 μM was injected into the cavity, the normal decay in P_j with time ceased within a few minutes and reversed direc-
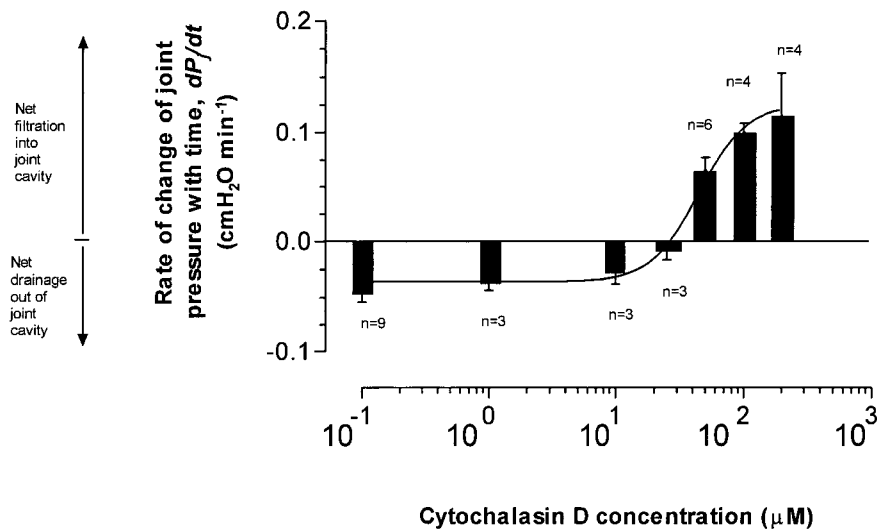
FIG. 2. Concentration–response relation for the effect of 1–200 μM cytochalasin D *in vivo* on dP_j/dt at t_{max} (mean $\pm$ SEM). Results for vehicle alone (zero concentration) are plotted at 10^{-1} μM. The fitted curve, with an EC_{50} of 45 μM, is described under Statistical Methods.

tion at 4.2 $\pm$ 0.9 min ($n = 14$) (Fig. 1). Pressure then increased with time, indicating a switch to net trans-synovial filtration of fluid into the joint cavity. The pressure gradually climbed higher than the initial spike of P_j caused by the injected volume. The rate of rise gradually slowed as P_j increased. Even so, in a few joints studied for 1 h, the pressure was still rising slowly at 60 min, despite the fact that P_j had reached 6–7 cm H_2O.

The rate of pressure change dP_j/dt depended on the concentration of cytochalasin D ($P < 0.001$, one-way ANOVA). Treatment with cytochalasin at $\geq$50 μM reversed dP_j/dt to positive values in all cases ($P < 0.05$, Bonferroni's test, $n = 14$), whereas none of the joints treated with 1–10 μM cytochalasin showed a reversal of the negative dP_j/dt ($n = 6$). The time at which the rate of pressure rise following $\geq$50 μM cytochalasin was steepest, t_{max}, was $\sim$10 min after the injection. The relation between dP_j/dt at t_{max} and the logarithm of concentration was sigmoidal (Fig. 2). Cytochalasin had little effect at 1 μM ($dP_j/dt = -0.033 \pm 0.010$ cm H_2O min^{-1}, pressure 0.53 $\pm$ 0.09 cm H_2O, $n = 3$) or 10 μM ($dP_j/dt = -0.028 \pm 0.010$ cm H_2O min^{-1}, pressure 0.30 $\pm$ 0.30 cm H_2O, $n = 3$), and the effect appeared to approach a maximum by 200 μM ($dP_j/dt = +0.115 \pm 0.039$ cm H_2O min^{-1}, pressure 4.9 $\pm$ 1.0 cm H_2O, $n = 4$). In the control joints dP_j/dt at times

corresponding to t_{max} was -0.046 ± 0.008 cm H_2O min^{-1} (pressure 0.38 $\pm$ 0.27 cm H_2O, $n = 9$). The estimated EC_{50} was 44.6 μM, with 95% confidence intervals of 36–55 μM.

Net trans-synovial flow was calculated as dP_j/dt multiplied by the compliance dV/dP_j at the corresponding pressure. Compliance values were taken from Knight and Levick (1982). For the control solution the average trans-synovial drainage rate was 3.9 μl min^{-1} at times corresponding to contralateral t_{max}. (Drainage is allocated a positive sign for consistency with previous work.) After treatment with 200 μM cytochalasin D the trans-synovial flows at t_{max} were in the range -6.6 to -12.5 μl min^{-1}, with the negative sign indicating net filtration into the cavity. Experiments *post mortem* showed that the microcirculation was responsible for the filtration into the cavity (see below). The effect of cytochalasin in joints was thus to initiate the rapid development of a joint effusion.

Although cytochalasin is known to act directly on endothelium (see Introduction), we also tested whether tissue protease activation might contribute to the response. The broad-spectrum protease inhibitor α_2-macroglobulin was added to 10 ml of 200 μM cytochalasin D in one experiment. The quantity of α_2-macroglobulin, 1 mg, was sufficient to inhibit >10 μg of trypsin activity or 10^4 BAEE units per milligram of
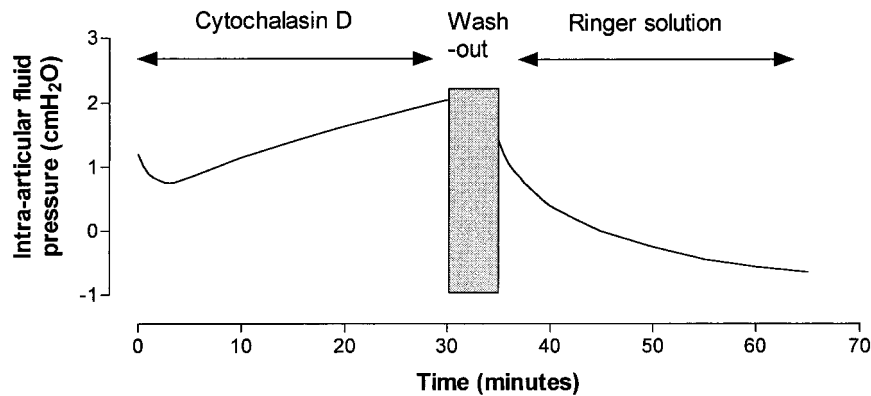
FIG. 3. Reversibility of the effect of 50 μM cytochalasin D on intra-articular pressure *in vivo*. The rising pressure indicated net filtration into the joint cavity in response to cytochalasin D. The falling pressure after washout of cytochalasin D with Ringer solution indicated net drainage out of the joint cavity.

protein. It had no effect, however, on the pressure-reversal response to cytochalasin D. Slope dP_j/dt reversed direction within 2 min and increased to +0.113 cm H$_2$O min^{-1}, which is equivalent to a net filtration rate of ~11 μl min^{-1} into the joint cavity.

Effect of Latrunculin B on Intra-articular Pressure in Vivo

In two joints latrunculin B (100 μM) was substituted for cytochalasin D in the standard protocol. Within 6 min of the latrunculin injection, P_j ceased to fall and began to rise. The rate of pressure increase, 0.033 cm H$_2$O min^{-1} (mean of two), was equivalent to a net filtration of 3 μl min^{-1} into the cavity. Since lantrunculin and cytochalasin both disrupt F-actin, but by different mechanisms, the results support the view that reversal of dP_j/dt is mediated by the breakdown of actin microfilaments.

Reversibility of Effect of Cytochalasin on Intra-articular Pressure and Filtration

The reversibility of the action of cytochalasin was investigated in three joints. After the effect of a 200-μl intra-articular injection of 50 μM cytochalasin D on dP_j/dt for 30 min was recorded, the cavity was washed out with Ringer solution (3 × 200-μl washes) and aspirated. The response of dP_j/dt to a 200-μl injection of Ringer solution was then recorded for another 30

min (Fig. 3). Following the initial cytochalasin injection, P_j began to rise at ~4 min and continued to increase until the washout at 30 min. After the washout and injection of Ringer solution to approximately the same starting pressure, the pressure decayed slowly with time, as in an untreated joint. The cytochalasin-induced positive dP_j/dt was thus readily reversed. Rapid reversal of drug action from positive dP_j/dt (net filtration) to negative dP_j/dt (net drainage) occurred in two joints, while in the third joint there was a partial reversal; i.e., dP_j/dt remained positive but its magnitude was much reduced.

Effect of Cytochalasin D on Intra-articular Fluid Pressure Post Mortem

To demonstrate the effect of rat dermal fibroblasts on interstitial fluid pressure, Berg *et al.* (2001) injected cytochalasin D *post mortem*; this prevented microvascular filtration from masking an increasing negativity of the interstitial fluid pressure. Since synovial fluid is in direct contact with the synovial interstitium, we looked for an analogous effect by measuring the response of P_j to cytochalasin *post mortem*. Experiments *post mortem* also tested the inference that the positive dP_j/dt after cytochalasin treatment is caused by microvascular filtration. Small volumes (100–200 μl) of 50–100 μM cytochalasin D ($n = 5$ joints) or 1 mM cytochalasin D ($n = 4$ joints) or Ringer solution ($n = 6$ joints) were injected into the joint cavity through the pressure
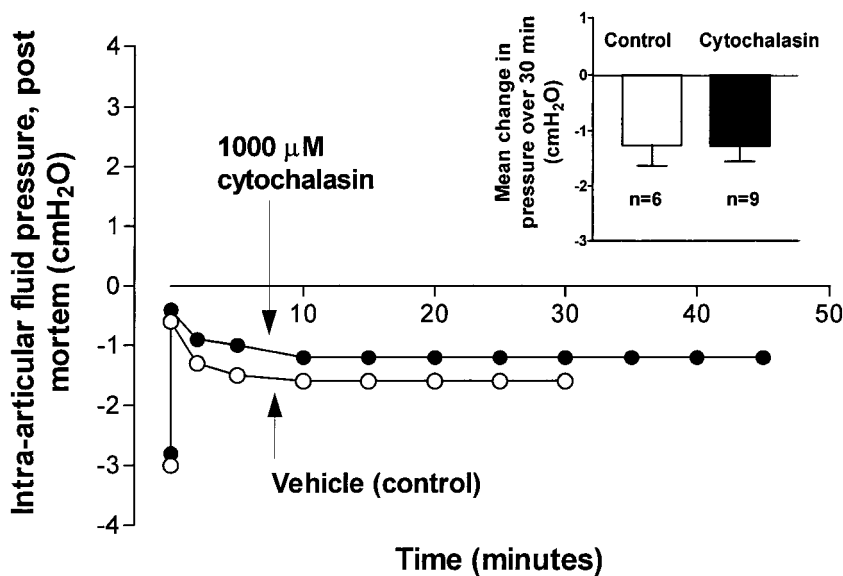
FIG. 4. Time course of intra-articular pressure in two knee joints *post mortem,* following an intra-articular injection of 120 μl of 1000 μM cytochalasin D solution or vehicle at time zero. The initial injection raised the endogenous, subatmospheric pressure almost to atmospheric pressure. The injectate volume was small in order to facilitate the detection of any progressive negative effect of cytochalasin on pressure. The experiment was carried out *post mortem* to eliminate microvascular filtration (cf. response *in vivo,* Fig. 1). (Inset) Mean fall in pressure from postinjection peak over a period of 30 min in nine joints injected with 50–1000 μM cytochalasin D and in six joints injected with vehicle (mean $\pm$ SEM bars; $P = 0.97$, unpaired *t* test).

line after cardiac arrest, and changes in P_j were followed for 30–60 min.

Figure 4 shows that, in marked contrast to the results *in vivo,* the effects of 1 mM cytochalasin D and control solution were not significantly different after circulatory arrest. After injection of the drug, P_j decayed slowly. The change in P_j with time closely paralleled that of the control solution, with P_j approaching an asymptote that presumably represented pressure equilibration with the adjacent synovial tissue. Since dP_j/dt after cytochalasin was negative *post mortem* (Fig. 4) yet positive *in vivo* (Fig. 1), it is inferred that positive dP_j/dt values *in vivo* depended on synovial microvascular perfusion.

In contrast to the observations of Berg *et al.* (2001) in rat skin, there was no evidence for the generation of extra negative pressures by cytochalasin in joints *post mortem.* The mean fall in P_j over the 30 min following the injection peak was -1.26 ± 0.38 cm H_2O for control solution ($n = 6$) and -1.28 ± 0.28 cm H_2O for cytochalasin D at 50–1000 μM ($n = 9$). The difference was not significant ($P = 0.97$, unpaired *t* test). The fall in P_j following injections of cytochalasin D at 1000 μM

was -0.71 ± 0.14 cm H_2O over 30 min ($n = 4$) and -0.63 ± 0.24 cm H_2O over 45 min. Over a similar period Berg *et al.* (2001) observed a change of approximately -2.7 cm H_2O in the cytochalasin-injected rat dermis, in contrast to $+0.6$ cm H_2O in saline-injected controls.

To test whether the volume of the injectate, 100–200 μl, might have obscured a developing negativity, the experiment was repeated in four more joints using 10-μl injections of 1 mM cytochalasin D delivered from a Hamilton syringe. The results were similar to those in Fig. 4; there was no evidence of the induction of increasingly negative pressures by cytochalasin. Mean pressure was -2.9 ± 2.1 cm H_2O immediately after the injection and -2.5 ± 1.4 cm H_2O at 30 min ($P = 0.9$, *t* test).

Effect of Cytochalasin D on Interstitial Fluid Pressure; Servonull Micropipette Results

It could be argued that measurements of P_j failed to detect the putative influence of synoviocytes on interstitial fluid pressure because pressure was recorded in

a contiguous space rather than in the interstitium itself. Calculations based on the relative compliances of the intra-articular and synovial interstitial compartments indicate that this is unlikely to be the true reason (see Discussion). To address the issue directly, however, we applied the servonull micropipette technique of Berg *et al.* (2001) to measure the interstitial fluid pressures (P_{if}) in the synovium and subsynovium *post mortem*.

Prior to the interstitial injection of test solution, the synovial and subsynovial interstitial pressures were slightly subatmospheric, with average values of -0.95 ± 0.37 cm H_2O ($n = 18$) and -0.81 ± 0.54 cm H_2O ($n = 18$), respectively. Repeated measurements of synovial and subsynovial P_{if} over 60 min in two joints that received no interstitial injections showed no significant change with time; none of the four regression slopes of P_{if} versus time was significantly different from zero ($P \geq 0.3$), indicating that the tissue hydration was not changing significantly.

The changes in mean P_{if} over 60 min following the injection of 1.5 μl of vehicle or 200–1000 μM cytochalasin D are shown in Fig. 5A (synovial P_{if}) and 5B (subsynovial P_{if}). Inspection indicated that neither synovial nor subsynovial P_{if} became consistently more subatmospheric in response to cytochalasin D. Analysis of the results for 0, 200, and 500 μM cytochalasin D showed no significant relation between P_{if} and time ($P = 0.8$, two-way ANOVA). Similarly, the regression slopes fitted to the mean synovial P_{if} versus time relation over 60 min for 200 μM (0.02 ± 0.01 cm H_2O min^{-1}, $n = 5$ joints) and 500 μM cytochalasin D (-0.04 ± 0.02, $n = 4$) were not significantly different from zero ($P \geq 0.14$); only after the control injections did the mean P_{if} drift slightly but significantly in a negative direction (slope -0.04 ± 0.01 cm H_2O min^{-1}, $P = 0.01$, $n = 5$). For subsynovium, none of the regression slopes for P_{if} versus time were significantly different from zero ($P \geq 0.18$). Similar results were obtained in two joints injected with 1000 μM cytochalasin D; there was no significant increase in the negativity of P_{if} with time ($n = 2$). By contrast, Berg *et al.* (2001), observed that 1000 μM cytochalasin D caused a progressive negative shift in P_{if} with time in rat skin, by approximately -2.7 cm H_2O over 60 min.
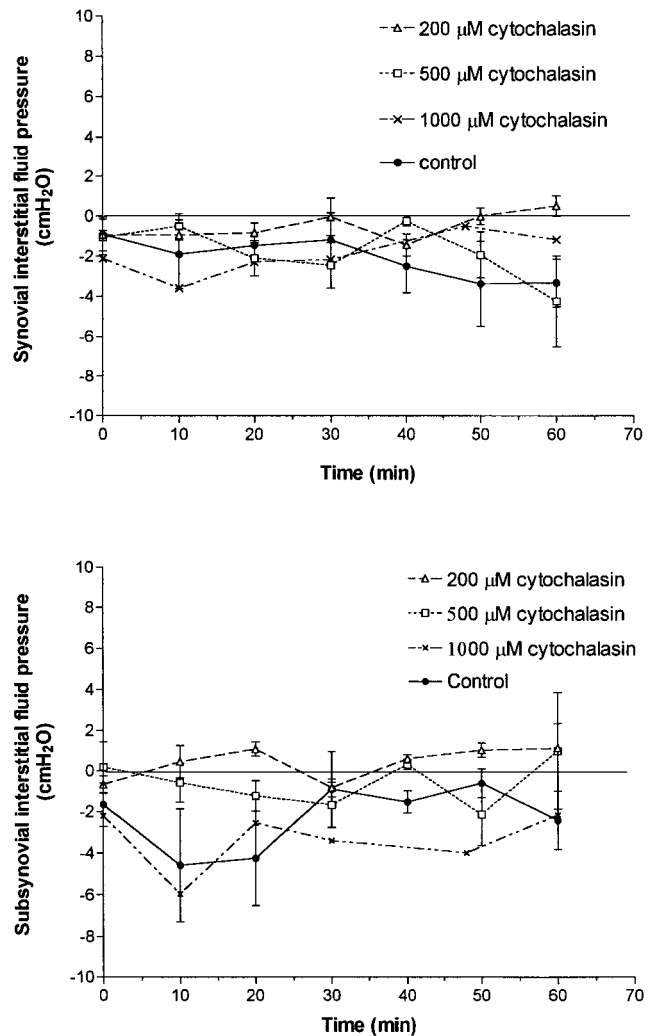

FIG. 5. Effect of cytochalasin D or control solution on interstitial fluid pressure in synovial lining (A; mean $\pm$ SEM) or subsynovial connective tissue (B), measured by servonull micropipette *post mortem*. Interstitial pressure was measured at $\sim$1 mm from the center of the injection site following the injection of an interstitial bolus of 1.5 μl of control solution ($n = 5$) or 200 μM ($n = 5$), 500 μM ($n = 4$), or 1000 μM cytochalasin D ($n = 2$). The measurement plotted at time zero was made immediately prior to the injection.

Half-Lives of Small Solutes in Joint Cavity in Vivo

Because no method was available to analyze the drug concentrations, intra-articular half-lives were determined for colored solutes of similar molecular size and diffusivity to latrunculin (395 Da) and cytochalasin D (508 Da); see Methods. The intra-articular half-lives of acridine orange (370 Da), patent blue V (582

Da), and Evans blue (963 Da) *in vivo* were respectively 13.8 ± 2.9 min ($n = 5$), 24.2 ± 2.7 min ($n = 2$), and 55.0 min ($n = 1$). The half-lives correlated significantly with molecular mass (Pearson's $r = 0.93$, $P < 0.001$). On the basis of these half-lives it is likely that the intra-articular concentration of the drugs fell substantially below the cited nominal concentrations over the 30-min study intervals *in vivo.*

Half-Lives Post Mortem

When clearance measurements were made in the contralateral joint *post mortem,* the intra-articular half-lives of acridine orange and patent blue V increased by 66–107%, to 22.9 ± 9.5 min ($n = 3$) and 50.2 min ($n = 1$), respectively. The difference between the paired results *in vivo* and *post mortem* indicated that small solutes are cleared partly into the microcirculation and partly by diffusion into the periarticular tissue ($P = 0.08$, $n = 4$, paired t test). Clearance from the cavity was calculated as intra-articular volume × 0.693/half-life. The difference between the clearance of acridine orange *in vivo* and *post mortem* was 55 ± 5 μl min^{-1}. Since acridine orange is a small, rapidly diffusible molecule that is soluble in both lipid and water (Budavari, 1996), and since fenestrated capillaries have a very high permeability to such solutes, its clearance by synovial capillaries is likely to be partly flow-limited. The above result may thus provide a minimal estimate of synovial microvascular perfusion, namely, ≥55 μl min^{-1} (see Discussion).

Effect of Cytochalasin on Plasma γ-globulin Permeation into the Joint Cavity

If the effect of cytochalasin on filtration is caused by endothelial gap formation, as inferred from studies *in vitro* (see Introduction), cytochalasin should also increase the permeation of large plasma proteins into the joint cavity. To test this, the net flux of native γ-globulin into a pair of joint cavities injected with control solution and 100 μM cytochalasin D solution, respectively, was analyzed; see Methods. Cytochalasin increased the net γ-globulin flux into the joint cavity from 0.73 mg h^{-1} (control) to 1.74 mg h^{-1}. When normalized by the γ-globulin concentration in plasma

from the marginal ear vein, the corresponding clearances of plasma globulin into the joint cavity were 63 μl h^{-1} (control) and 133 μl h^{-1} (cytochalasin-treated). A more extensive study using labeled bovine albumin showed that intra-articular cytochalasin increases the plasma clearance of albumin into rabbit knee joints (Poli and Levick, 2000).

DISCUSSION

The principle positive findings were that both cytochalasin and latrunculin caused large increases in pressure and fluid filtration into the joint cavity *in vivo* and that cytochalasin increased the permeation of plasma γ-globulin into the joint cavity. The EC$_{50}$ value for these effects was higher than that for cultured endothelia *in vitro,* but this may be due partly to the short intra-articular half-life of the drug. Even so, near maximal effects were observed at 100–200 μM cytochalasin, which is almost an order of magnitude lower than the concentration of 1 mM used by Berg *et al.* (2001) to demonstrate statistically significant changes in rat skin.

Endothelial Origin of Increased Permeation of Macromolecules and Fluid

The increased permeation of γ-globulin into the joint, and of intravascular labeled albumin (Poli and Levick, 2000), indicates a major increase in microvascular permeability. This may be due to endothelial gap formation, which is a well-documented response *in vitro* (Shasby *et al.,* 1982; Alexander *et al.,* 1988; Haselton *et al.,* 1989; Holda and Blatter, 1997; Kevil *et al.,* 1998). In support of this, intravascular injections of Monastral blue *in vivo* demonstrated that intra-articular cytochalasin induces spotty leakage in synovial microvessels (Poli *et al.,* 2001). The high filtration rates could likewise be due to endothelial gap formation; gaps are expected to raise the endothelial conductance and probably reduce the albumin reflection coefficient, which is normally ≥0.8 in synovial capillaries (Knight *et al.,* 1988). A spatially distributed model of trans-synovial flow predicts substantial increases in

filtration into the joint cavity in response to moderate reductions in effective colloid osmotic pressure across the synovial endothelium (Levick, 1991; Fig. 3C).

The filtration rates induced by cytochalasin may represent a substantial fraction of the synovial plasma flow. Synovial microvascular perfusion is an important parameter for which there is scanty information. The estimate of ≥ 55 μl min^{-1}, based on acridine orange clearance *in vivo* versus *post mortem,* is of similar magnitude to that inferred from the mean synovial capillary volume (Levick and McDonald, 1989a, b) divided by mean transit time (Funk *et al.,* 1995), namely, 64–128 μl min^{-1} in the rabbit knee; the latter estimate corresponds to a plasma flow of 45–90 μl min^{-1} for a dynamic microvascular hematocrit of 0.3. If the synovial plasma flow is, then, of the order 55 μl min^{-1}, the filtration rates into the joint cavity of 6.6–12.5 μl min^{-1} in response to 200 μM cytochalasin D represent filtration fractions of 12–23%. Although the filtration fraction would be reduced if cytochalasin D raised the synovial blood flow, a pilot study using N_2O wash-in/washout and an intra-articular N_2O-sensitive electrode provided no evidence of a hyperemic response to cytochalasin.

Since the primary action of cytochalasin and latrunculin is to disrupt actin microfilaments, the results indicate that F-actin is an important structural element in the fenestrated endothelial barrier *in vivo.* This may be related to the fact that the junctional proteins, such as the cadherin–catenin complex, are restrained spatially in the fluid lipid bilayer by linkages through α-actinin to the actin cytoskeleton (Dejana *et al.,* 1995). The results do not exclude the formation of transcellular as opposed to intercellular gaps (Michel and Curry, 1999). Cytoskeleton disassembly can have additional, less direct effects (Janmey, 1999). For example, F-actin disassembly in endothelium but not fibroblasts impairs capacitative Ca^{2+} entry and impairs receptor-mediated Ca^{2+} entry in both cell types (Holda and Blatter, 1997; Ribeiro *et al.,* 1997).

Rapidity and Reversibility of Drug Action

The turnover of F-actin is rapid in many cultured cell lines (Symons and Mitchison, 1991), and cytochalasin *in vitro* increases the permeability of an arterial endothelial monolayer to dextran by fourfold in 30 min (Kevil *et al.,* 1998). The impressively rapid effects of cytochalasin observed in the present experiments *in vivo* (Fig. 1) may be due to a very fast turnover of F-actin in the fenestrated endothelium. Studies *in vitro* show too that the cytochalasin-induced hyperpermeability of monolayers can be reversed within an hour (Shasby *et al.,* 1982; Haselton *et al.,* 1989). The present experiments, which had a time resolution of minutes, showed that reversal begins within minutes of drug washout *in vivo* (Fig. 3). This may again be the result of a rapid turnover and rebuilding of filamentous actin in synovial endothelium.

Does Cytochalasin Treatment Affect the Synovial Interstitial Matrix Too?

Transport between the circulation and joint cavity involves two barriers in series, the endothelium and synovial intercellular matrix, but several lines of evidence point to the endothelium rather than the interstitial layer as the primary responder, as follows. (i) The effect of cytochalasin on P_j was prevented by circulatory arrest (Fig. 1 and 4). (ii) Endothelium is the chief site of resistance to plasma protein permeation, and the reflection of plasma protein by the interstitial layer is negligible (Scott *et al.,* 2000a). (iii) Studies using Monastral blue show the development of endothelial leaks (Poli *et al.,* 2001). (iv) Cytochalasin increases the permeability of endothelial monolayers to albumin (Shasby *et al.,* 1982; Biffl *et al.,* 1995; Lee *et al.,* 1998).

Nevertheless, the findings of Berg *et al.* (2001) in rat skin introduced the possibility that cytochalasin treatment might also affect the interstitial matrix. If the synoviocyte actin cytoskeleton "braces" the matrix through integrin–collagen linkages, as postulated in rat skin, then cytochalasin treatment could in principle induce a more negative interstitial fluid pressure. The servonull micropipette measurements of interstitial pressures *post mortem* showed, however, that cytochalasin had no more effect on synovial or subsynovial interstitial fluid pressure than did control solution. Nothing resembling the extra -2.7 cm H_2O interstitial pressure observed in rat skin *post mortem* (Berg *et al.,* 2001) was detected in the present study. Likewise the

effect of cytochalasin or latrunculin on intra-articular pressure *post mortem* was no different from that of vehicle (Fig. 4), even when the injectate volume was only 10 μl. The reasoning behind the intra-articular study was that any increase in the swelling tendency of synovial interstitium will cause the absorption of some intra-articular fluid *post mortem* and thus reveal itself by a fall in intra-articular pressure. The extent to which intra-articular pressure can be expected to mirror changes in periarticular swelling pressure depends on the relative volume and compliance of the synovial cavity and the periarticular tissue. Calculations using estimates of compartment volumes and compliance from Knight and Levick, (1982), Levick and McDonald (1989a, b), Price *et al.* (1996), and Scott *et al.* (2000b) indicate that if the synovium and subsynovium developed an extra -2.7 cm H_2O swelling pressure, the intra-articular pressure would change by -1.6 cm H_2O, given an injection volume of 10 μl at subatmospheric joint pressure (the optimal conditions for detecting the putative effect). Despite its inferred detectability, no such response was seen. The results, therefore, provided no support for the hypothesis that in rabbit synovium the actin cytoskeleton of the fibroblasts (synoviocytes) directly influences the matrix fluid pressure.

To summarize, the study showed that endothelial F-actin has a major role in determining the normal synovial microvascular resistance to fluid filtration and plasma γ-globulin permeation. F-actin disruption is thus a potential factor in the link between pro-inflammatory mediators and the formation of arthritic joint effusions. The results provided no support for the hypothesis that synoviocyte F-actin also influences the swelling tendency of synovial matrix and hence its hydration and permeability.

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