The human knee: A window on the microvasculature

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Abbreviations: S, Serum; SF, Synovial Fluid; IF, Interstitial Fluid; P, Plasma; OA, Osteoarthritis; BCP, Basic Calcium Phosphate; RA, Rheumatoid Arthritis; NSAID, Non-steroidal Anti-inflammatory Drugs; Cl, Clearance; C, Concentration; nm, nanometer

In synovial joints, the lining cells do not have tight junctions with their neighboring cells and they have no underlying basement membrane. Therefore, the synovial fluid within the articular cavity is continuous with the interstitial fluid of the synovial intima. These features, combined with ready access to the space via arthrocentesis, permit quantitative studies of microvascular function in the knees of unanesthetized, volunteer, human subjects both with and without chronic arthritis. This brief article reviews the principal findings of such work over ~40 years at the University of Washington. Examined variables include bidirectional fenestral diffusion of small solutes, effective blood flow, lymphatic drainage, and endothelial pore size and permeability. The latter work introduced a new method using gel filtration chromatography of paired synovial fluid (SF) and serum (S) to obtain essentially continuous SF/S ratios over a range of radii between 1 and 12 nanometers.

“No apparent barrier exists separating the articular cavity from the intercellular spaces of the synovialis. . .” (Bauer, Ropes and Wayne, 1940).¹

Background

In human knees, the proteins of synovial fluid (SF) are essentially the same as those in the interstitial fluid (IF) of the adjacent synovial tissue.² Unlike other body cavities, such as the peritoneum or the pericardium, the synovial lining is not mesothelial. Thus, the junctions between the macrophage-like type A cells and the fibroblast-like type B cells at the surface are not tight and there is no underlying basement membrane.³ (Fig. 1) As a result the well mixed SF and IF proteins are derived from the same microvascular bed and are ultimately cleared at the same rate by the same tissue lymphatics.⁴ Because of these properties, and the ready accessibility of SF through simple aspiration, the knee provides a unique opportunity for studies of microvascular function. Notably, the fluids are confined by an investing capsule, their total volume

remains essentially constant, no other organs share their space, and no vascular bed supports that space other than the fenestrated vessels of the synovium.⁵ These features provide obvious advantages over traditional study sites where access to interstitial fluid required surgical intervention, yielded fluid admixed from several regional tissues, and largely precluded investigations of human subjects. From a physiological perspective, there are additional advantages in studying living perfused tissues rather than films of cultured endothelial cells *in vitro*.

Small Molecules

Very few of these advantages were obvious when, as a student of gouty arthritis (arthritis induced by the extracellular crystals of sodium urate that are pathognomonic of gout), I chose to examine the manner in which urate ions move back and forth between plasma (P) and SF across the synovium of normal human knees. At rest, the small molecules of P and SF are fully equilibrated.¹ To examine the kinetics of exchange between them, it is necessary to disrupt that equilibrium and then study the reequilibration process over a relatively short time period. This is readily done by instilling isotonic saline and then withdrawing and analyzing serial samples. Jayson and Dixon had shown that the articular cavity of the knee would accommodate 30 ml of saline with little change in pressure and only modest discomfort at the locally anesthetized injection site.⁶ Goetzel et al had taken advantage of this property and used serial aspirates of injected saline to study the kinetics of oxygen consumption and lactate production.⁷ It seemed logical to adapt this model to measure declining concentrations of injected, ¹⁴C-labelled urate together with rising levels of endogenous urate. Ultimately, exogenous ¹⁴C labelled urea and glucose were also assessed together with their endogenous, unlabeled counterparts, ³H clearance was included in all studies, several additional solutes from plasma (including total protein) were measured, and benzyl alcohol was examined since this bacteriostatic agent was included in our 30ml vials of 0.9% saline.⁸

The experiments went well. Rates of transynovial exchange were inversely related to molecular size, and the labelled urea and urate left the joint at rates which were essentially the same as the entering rates for endogenous urea and urate.

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Figure 1. Scanning EM view of canine synovium seen en face. Note the broad gaps between lining cells revealing collagen fibers within the interstitial matrix. (From Kondoh ³, with permission)

Thus, the kinetics were clearly those of bidirectional diffusion and we concluded (as did the British vascular physiologist Rodney Levick in his separate analysis) that the limiting diffusion path for small molecules was that through the interstitial tissue of the synovium rather than the porous endothelial fenestrae.^{9,10}

Three important exceptions were found to the concept of simple interstitial diffusion. 1. Physiologic glucose entered more rapidly than labelled glucose left. We felt this was probably due to a carrier-mediated transport system in the synovial lining cells. No other specific transport system has been recognized in the synovium. 2. Tritiated water left more slowly than its size would indicate. We interpreted this as the result of full equilibration between P and IF during each passage through the microvessels and therefore used the clearance of tritiated water to estimate the effective synovial plasma flow. 3. Benzyl alcohol, though a relatively large molecule, left the joint at rates comparable to those of tritiated water. We felt that this solute was able to equilibrate fully with P because its solubility in fat allowed it to diffuse through as well as between synovial lining cells just as respiratory gasses are known to move across the vascular endothelium.

Urate, the molecule which instigated this work, obeyed the rule of size dependence in 51 normal knees by entering at rates which were $49 \pm 1\%$ of those at which tritiated water left.⁹ This difference led to the differential diffusion hypothesis to explain the well-known predilection of gouty arthritis for ossearthritic first metatarsal phalangeal joints.¹¹ Thus, when these damaged joints swell during exercise, water will enter faster and urate levels will be lower in SF than in P. The two fluids reequilibrate as exercise continues, but at subsequent rest the process is reversed when water leaves faster than urate. Then, the SF levels rise above those of P with a resultant focal hyperuricemia which promotes crystallization with secondary podagra. This sequence fits well with the typical pattern of nocturnal attacks after an active day. Although still unproven, this hypothesis remains the most plausible explanation for this classic predisposition of gout for the base of the great toe.¹²

Iodide and protein

Many previous investigators had injected radiolabelled, gamma-emitting compounds into both normal and diseased human knees and followed their clearance with external counters.¹³ This technique yields strikingly, log-linear plots which then can be expressed as rate constants (in min^{-1}) or as half-lives (in minutes or hours). However, previous workers had not considered the volume occupied by the labelled substance. That can be determined readily by measuring the total counts injected at the outset, correcting this total to the final counts remaining (by using the external counting data to subtract the amount cleared), measuring the concentration in a final aspirate, and then calculating the volume of distribution by mass balance.⁵ Once the volume is known, one can evaluate the effective synovial blood flow as well as the rate of lymphatic drainage.

These useful parameters are obtained by multiplying the calculated volume (ml) by the measured clearance constants (min^{-1}) to obtain clearance values in ml/min. Because $^{131}\text{I}^-$, like tritiated water, appears to equilibrate fully with the perfusing plasma stream, that product represents a measure of the effective synovial plasma flow. When proteins of different size, labelled with different isotopes of iodine, are injected together, they are cleared at the same rate.⁴ This lack of size selectivity indicates a common, bulk-flow pathway consistent with lymphatic drainage, and the clearance rate of any specific protein, usually albumin, may be used as a measure of the rate of lymph flow.⁵ (Fig. 2)

Because recovery of the requisite final SF samples from normal knees is uncertain, these isotopic tools have not been used in normal subjects, but they have been informative in patients with arthritis. There, studies in Seattle and in London found the clearance of albumin, i.e. the rate of lymph flow, to be essentially twice as fast from the average rheumatoid knee as it was from the studied OA knee.^{5,14} (Table 1) It must be noted that knees cho-

Figure 2. Clearance of free ^{131}I and radiolabelled albumin (RISA) from a rheumatoid knee over 3 days. External counts yield linear clearance values for both markers. After a brief equilibration, counts in synovial fluid aspirates parallel the external counts. (From Wallis et al⁵)

Table 1. Clearance of albumin from rheumatoid and osteoarthritic knees

	Seattle ^{5a}		London ¹⁴	
	n	ml/min	n	ml/min
Rheumatoid	11	0.071 +/- 0.028	8	0.07 +/- 0.01
Osteoarthritis	9	0.039 +/- 0.030	8	0.04 +/- 0.01

sen for this work had significant effusions, perhaps related to apatite (BCP) crystals.¹⁴ Thus, these knees were obviously inflamed and the more common, non-effused OA knee is quite likely to be cleared at even slower rates.

These considerations are particularly relevant to the common practice of measuring concentrations of potential biomarkers in SF to interpret the pathophysiology of both synovial and cartilaginous disease.^{15,16} Clearly, the presence of such molecules in SF does reflect their release into the joint space, but their concentration depends as much on their clearance as it does on their release. Thus, for instance, a 3g/dL concentration of albumin (perhaps the most basic of all biomarkers) in both OA and RA aspirates does not mean the same vascular “leak” into these joints. Instead, it indicates an influx in RA that is twice that of OA (since the RA clearance is approximately twice that of OA). This principle is fundamental to all studies of biomarker concentrations in SF aspirates as they compare individuals or conditions with established standards or with each other. Concurrent studies of albumin or of another plasma protein of choice are relatively straightforward and offer an essential tool when biomarkers are used to quantify disease mechanisms and responses to therapy.

Pharmacokinetics

A related clinical concern involves the delivery of pharmacologic agents to arthritic joints.¹⁷ Many antibiotic and anti-inflammatory agents are known to be highly protein bound in vitro, and this property has raised understandable concern regarding their ability to reach articular targets. Naproxen, for instance is ~99% bound, the effective plasma flow to rheumatoid knees is ~2 ml/min¹⁸, and the volume of SF plus IF may be ~100ml. Thus, it might seem that only ~1% of the free drug would be available and adequate delivery of naproxen would not be possible, but that assumption would be wrong. In fact, a compartmental analysis of pharmacokinetic data reveals that ~23% of this drug enters the joint with each passage through the microvasculature. Similar kinetics were found for 7 other non-steroidal anti-inflammatory drugs (NSAIDs). We believe that these data imply that the NSAID-albumin bond is of low avidity despite its high affinity in static testing. As free-drug is delivered, it is promptly replaced from the bound-drug store and this happens over and over during each passage through the microvasculature. This interpretation sees NSAID transport as analogous to that of oxygen which is highly bound to hemoglobin within the circulation but diffuses readily down concentration gradients when they are encountered in peripheral tissues.

Permeability

In our simple model, the synovium of the knee is seen as a single, homogenous tissue, supplied by a uniform fenestrated microvasculature, cleared by a non-selective lymphatic drain, and contained in an impervious investing capsule. Within this space, the synovial fluid (accessible by needle) equilibrates fully with the interstitial fluid of the synovial tissue. Each plasma protein enters the SF of the knee at a single rate but those individual rates vary inversely with molecular size. As a result, the concentration ratio, SF/S, varies inversely as well. Small proteins, such as insulin, pass through the abundant fenestrae and their SF/S ratio is ~1. Mid-sized proteins are too big for the fenestrae and are much more limited as they pass between cells through the “small pores” at the endothelial junctions. Very large proteins can enter the joint only when these junctions open widely or when the proteins can be carried through the cells in vacuolar structures known as caveolae. These concepts are in accord with classic pore-based concepts of microvascular permeability.¹⁹

To test this model and to determine the size of the apertures, we chose not to determine SF/S ratios of individual proteins as others have done in the past.²⁰ Instead, we used gel filtration chromatography of the entire serum and synovial fluid proteomes in paired samples at 0.5nm increments over a continuous range of sizes (from 1.0nm to 12 nm).²¹ Thus, these analyses included the great majority of all S and SF proteins. Samples were obtained from 17 “normal” knees of 10 recently deceased individuals who had no known articular disease and from 16 patients with a variety of rheumatic diseases who were seen in the rheumatology clinic.

At equilibrium, the rate of ingress from the serum into the joint equals the rate of egress from the joint into the lymphatics and then ultimately back into the serum. The amount of that protein transported in each direction (mg/min) is the product of its concentration (mg/ml) and its clearance (ml/min): $Cl_{sf} \times C_{sf} = Cl_s \times C_s$. Since representative values for Cl_{sf} , C_{sf} and C_s were known, it was possible to solve for Cl_s at each size interval and to

Figure 3. Entry of serum proteins into normal knees as a function of molecular radius in nanometers (nm). Rapid entry of small proteins (radius less than 1.75 nm) is ascribed to microvascular fenestrae. Larger proteins (radius up to 8.5 nm) are thought to enter through the “small pores” at interendothelial clefts. The largest proteins enter through “large pores” by an undetermined mechanism. (From Simkin and Bassett²²)

Table 2. Dimensions of and flow through synovial microvascular pores

	Pore Radius (nm)		Vascular Efflux ($\mu\text{L}/\text{min}$)		Flow ratio	Filtration Fraction(%)	
	Normal	Effused	Normal	Effused	Effused/normal	Normal	Effused
Fenestra	1.75	3.5	1.74	22.0	12.6	0.087	1.10
Small pore	8.6	8.5	1.50	9.1	6.1	0.075	0.46
Large pore	40	36	0.24	15.5	64.6	0.012	0.78

(From Simkin and Bassett²²).

assess permeability from the full plots of Cl_s against molecular size. **Fig. 3** Our data agreed well with the 3-pore model ($r = 0.992$ in normal knees and 0.980 in arthritic knees).

The fenestral pore radius, 1.75nm , had not been quantified by previous investigators using the methods then available. This radius increased to 3.5 nm in effused knees and the filtration fraction increased by 22 fold; effects we tentatively assigned to damage to the glycolcalyx induced by inflammatory cells and/or cytokines. Traditionally, the endothelial surface was seen as a “cobblestone” of smooth adjoining cells, but that picture has changed. Now, with appropriate fixatives, electron microscopy shows that surface to be more like a grassy lawn with the “grass” being proteoglycan filaments, as much as $50\text{-}500\text{ nm}$ in length, that intrude into the vascular lumen. Over fenestrae, where most small molecules enter and leave the vasculature, the filaments are even longer and bushy in appearance.²² Collectively, these features are known as the glycocalyx, and the potential role of the glycocalyx as a determinant of permeability is now a major focus of microvascular physiology. We assume that the glycocalyx in fenestrae of synovial capillaries resembles those of other fenestrated beds, but to our knowledge the synovium has not yet been examined with appropriate techniques of tissue fixation.

The “small pore” radius of 8.6nm in normal knees and 8.5nm in effusions was twice the best previous estimate (4nm)²⁰. Of note, the diameter of this gap (17nm) is within range of the 20nm intercellular aperture found in morphologic studies and this pore remained essentially unchanged in our studies of effused knees.²³ This stability is surprising since many of those knees were markedly inflamed, and the glycocalyx is known to be vulnerable to the cytokines of acute and chronic inflammation. This finding leads us to suggest that the glycocalyx may not be a critical determinant of vascular permeability for molecules in this size range. Although the radius of this pore did not change, its rate of filtration increased by more than 6 fold while the large pore increased, as expected, by a much greater degree. (**Table 2**) Unfortunately, the effusions studied came from patients whose disease was in a variety of stages and whose diagnoses varied too widely to permit useful clinical comparisons within the group.

A small, but consistent, dip in SF/S was found at a radius of 4.5nm in normal knees. This is the radius of haptoglobin; a protein that has been thought to be excluded from SF by its strong negative charge. Clearly, the charge of other, less prevalent anionic proteins may also limit their permeability, but the excellent fit of our data suggests that any such contribution must be minor. Conversely, any protein synthesized in synovium or cartilage and released directly into the joint would be expected to cause a peak

in the curve, but no such peaks were observed. Again, the lack of such peaks does not exclude local synthesis, but it does indicate that synovial synthesis, when present, must be small in comparison to the large and steady influx of proteins from the plasma.

The Ischemic Knee

Articular ischemia is a classic manifestation of rheumatoid arthritis that (along with leg ulcers and rheumatoid vasculitis) is now seen rarely. In its most extreme form, the synovial fluid findings included pH values less than 7.0 , elevated lactate concentrations, and strikingly low glucose levels.²⁴ High counts of white blood cells (predominantly PMNs) were also present and florid synovitis was seen in tissue biopsies. Thus, such joints are marked both by biochemical evidence of ischemia and by pathologic evidence of inflammation. In concert with the evidence of ischemia, effective blood flows as measured by clearance of both tritiated water and radioactive iodide was lower in rheumatoid knees than in knees with other causes of arthritis.^{25,26} Moreover, the temperature of rheumatoid knees correlated directly with the iodide clearance. Rheumatologists are trained to associate active inflammation with the “hot” joint, but in this case that precept may not hold. To date, the most plausible explanation for this apparent discontinuity comes from findings of a morphometric study that normal synovial capillaries lay close to the surface of the tissue whereas rheumatoid synovial capillaries were deeper and more widely spaced within the greatly thickened intima of these joints.²⁷ This finding fits with the previous conclusion that effective delivery of small solutes from the plasma to the joint space depends more on passage through the interstitial intima than on transport across the microvascular wall.⁸ Heightened consumption of oxygen and glucose by inflammatory cells clearly contributes, but it has not yet been possible to dissect the transport from the metabolic factors.

Conclusion

We find the synovial interstitium to be the principal impediment to diffusive delivery of small molecules while the microvascular endothelium is the most significant barrier to transport of proteins between plasma and synovial fluid (SF) in the knee. Recognition of these factors and access to SF in the knee permits quantitative studies of microvascular function at this site in normal people and in the many forms of gonarthritis (arthritis of the knee). We recognize that our studies were limited to the knee

and that findings may differ in tissues with continuous capillaries and in those with other fenestrated beds.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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