Endothelial dysfunction and decreased vascular responsiveness in the anterior cruciate ligament-deficient model of osteoarthritis

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PGE2 had a biphasic response that decreased perfusion at 10⁻¹⁴ mol and was dilatory at higher concentrations. Substance P caused a biphasic response that was dilatory from 10⁻¹⁴ to 10⁻¹¹ mol and constricting at higher doses. In ACL-deficient knees, ACh, bradykinin, histamine, and SP induce vasodilation and inhibit platelet aggregation (42). Bradykinin stimulates vascular responses through the B2 receptor (42, 51). The B2 receptor is a G protein-coupled receptor that is constitutively expressed in the vasculature and is capable of increasing intracellular calcium concentrations in both endothelial cells and vascular smooth muscle cells. Activation of the B2 receptor in endothelial cells results in the release of two vasoactive compounds: prostaglandin I₂ and NO (42, 51). Prostaglandin I₂ activates the inositol phosphate receptor to induce vasodilation and inhibit platelet aggregation (42).

The vasoactive effects of histamine are tissue dependent. Chiba and Tsukada (13) have shown that removal of the vascular endothelium almost completely removes the dilatory effect of histamine, whereas contractile effects remain. Histamine-induced vasodilation appears to be mediated only by the H₁ receptor, whereas both the H₁ and the H₂ receptors mediate the contractile effects (13). Similarly, substance P-induced vasodilation is endothelium-dependent, mediated through the neurokinin (NK) type 1 receptor. In the absence of an intact functional endothelium, substance P induces vasoconstriction. The NK type 2 and NK type 3 receptors do not appear to have any function in vasoregulation (45).

PGE₂ mediates both vasoconstrictor and vasodilator responses in different tissues. Four receptor subtypes have been cloned and are designated E1, E2, E3, and E4, all of which are G protein-coupled receptors. The E1 and E3 receptors induce vasoconstriction, and conversely, the E2 and E4 receptors induce vasodilation (14). The E1 receptor functions through a G₃ pathway to cause an increase in intracellular calcium and is highly active in the vas deferens and iliac smooth muscle (15). The E3 receptor is coupled to a G₁ pathway and causes an
inhibition of cAMP accumulation (50). The E2 and E4 receptors are coupled to Gi pathways and cause an increase in intracellular cAMP (15, 32, 33). These two receptors are differentiated only by their affinity for different selective agonists, butaprost and (PGE1)-OH respectively (15, 32).

Response to shear stress has become a noninvasive diagnostic tool to assess endothelial function in patients (54). Following a period of occlusion, postocclusion arterial flow is increased, and stretch-activated receptors induce activation of eNOS to produce NO and induce vascular smooth muscle dilation (47, 56). Diminished response to shear stress is also indicative of endothelial dysfunction. (27, 54).

Under chronic inflammatory conditions, the vasculature may also become inflamed (20, 49). An inflamed vasculature may become involved in proinflammatory processes and may contribute to the chronicity and severity of the pathology. Examination of vascular functioning can indicate the condition of the vasculature in this injury OA model, and it may aid in understanding the progression of OA and the injury response of the MCL in this model. The questions addressed by these studies are: what are the responses of the MCL vasculature and the endothelium in a chronic degenerative OA model with some aspects of inflammation, and if these responses are abnormal, do they implicate any other pathological conditions that may be involved?

METHODS

Twenty-four skeletally mature 1-yr-old New Zealand White rabbits (4.5–6.5 kg) were used in two cohort groups: unoperated control (n = 12) and 6-wk ACL transected (n = 12). Both cohorts were further divided into groups of six for blood flow assessment. Previous studies have shown no significant effect of sham operation on tissue blood flow at 6 wk (5–7); therefore, a sham-operated group was omitted, and normal controls were used. Rabbits were kept on a 12:12-h light-dark cycle, and they were fed standard laboratory chow and tap water ad libitum. All animals were treated and maintained according to the Canadian Council on Animal Care Guidelines under a protocol approved by the Faculty of Medicine Animal Care Committee.

ACL transection. Rabbits were given 0.18 ml of acepromazine maleate (Ativan) intravenously and anesthetized with halothane (2–5%, 1.0 l/min O2). An anterior tibial draw test was performed before surgery to ensure that there was no preexisting ACL injury condition. An anterolateral surgical approach was used. The patellar fat pad was retracted, the ACL was isolated using a hooked probe, and the ligament was cut using a no. 11 surgical blade. A second anterior tibial draw was performed to ensure that the transection was complete. Following surgery, rabbits were treated with standard antibiotics and analgesics and allowed to resume normal cage activity for 6 wk.

Blood flow imaging. Under halothane anesthesia (1–2%, 1 l O2/ min), the MCL was surgically exposed. Overlying fascia was carefully dissected away without damaging the network of blood vessels supplying the MCL. Blood flow was measured using laser speckle perfusion imaging (LSPI). Full details of this instrument can be found in previous publications (5, 17, 18). Briefly, a 635-nm laser source is connected by a fiber-optic cable to the LSPI instrument head, which also contains a black and white charge-coupled device camera with a close-focus imaging zoom lens. The instrument head was placed 23 cm directly above the MCL for a uniform and simultaneous illumination of the entire region of interest, and the lens focus was adjusted until the region of interest filled the camera field of view. Exposure time was set at 15 ms. LSPI camera output was fed directly to a live monitor for continuous video of the laser-illuminated tissue and to a computer for simultaneous capture and digitization of speckle images. High-resolution digital images were processed using custom LSPI algorithms to produce quantitative color-coded perfusion maps of tissue blood flow (Fig. 1). Images were analyzed to determine average perfusion values within a user-specified region defined by the anatomical borders of the MCL. Figure 1 shows examples of color-coded perfusion images of the MCL and the defined analysis regions used to produce the perfusion values. Results are presented as mean perfusion units (PU) ± SE.

Fig. 1. Laser speckle perfusion images of rabbit medial collateral ligament in a control animal (a) and an experimental animal 6 wk after transection of the anterior cruciate ligament (b). Margins of the medial collateral ligament are outlined in black. Perfusion for each medial collateral ligament following topical application of saline is given at the bottom right of each image. Enlarged views of the control medial collateral ligament shown in a are shown in c and d, demonstrating vasoconstriction (c; substance P 10−8 mol) and vasodilation (d; substance P 10−12 mol) of vessels supplying the medial collateral ligament (arrows). Also note the color shift in area X and similar regions, indicating altered microcirculation. PU; perfusion units.
**RESULTS**

Six weeks following ACL transection, gross morphological changes were observed. Synovial hyperplasia, capsular thickening, MCL scarring, and bucket handle medial meniscal tears were found in all ACL-deficient knees. Cartilage fibrillation and osteophyte formation were not present. Knee joint diameter of ACL-deficient knees was measured with calipers and found to be significantly increased compared with control knees (29.4 ± 0.3 vs. 22.1 ± 0.2 mm; \( P < 0.001 \)).

The mean perfusion of control MCLs was 7.41 ± 1.22 PU. In accordance with our previously published results (41), perfusion of the MCL was significantly increased in ACL-deficient knees to a mean of 18.32 ± 1.76 PU (\( P \leq 0.006 \)). Representative LSPI of the MCL in control and ACL-deficient knees are shown in Fig. 1, a and b. Topical application of saline did not alter blood flow in either control knees (Fig. 1a) or in ACL-deficient knees (Fig. 1b). The resolution of the LSPI was sufficient to detect drug-induced vasoconstriction and vasodilation of the small vessels supplying the MCL (Fig. 1, c and d).

**Drug responses.** ACh increased perfusion in a dose-dependent manner in the exposed MCL vasculature. Maximal response to ACh in control knees was observed at \( 10^{-8} \) mol where perfusion was increased by 43.1 ± 7.3%. The ACh-induced dilatory response was not observed in ACL-deficient knees. ACh reduced perfusion in ACL-deficient knees by −3.9 ± 0.2% at \( 10^{-8} \) mol (Fig. 2).

Similarly, bradykinin increased perfusion to the MCL vasculature in control knees in a dose-dependent manner. Maximal dilation was observed at \( 10^{-9} \) mol. At this concentration, bradykinin increased perfusion by 32.3 ± 3.3%. The dilatory response to bradykinin was not observed in ACL-deficient knees, and at \( 10^{-9} \) mol, bradykinin decreased perfusion by −9.8 ± 3.3% (Fig. 3).

Exposure of the MCL tissue to histamine led to the dose-dependent induction of vasodilation in control knees with a maximal response obtained at \( 10^{-9} \) mol, which increased perfusion by 51.3 ± 5.6% (Fig. 4). The histamine dilatory response was altered in the ACL-deficient knee, where \( 10^{-9} \) mol of histamine decreased perfusion by −7.8 ± 4.4%.

In control knees, exposure to exogenous SP led to dose-dependent increases in perfusion at concentrations from \( 10^{-14} \) to \( 10^{-11} \) mol and to decreases in perfusion at higher concentrations (Fig. 5). Maximal dilation was observed at \( 10^{-12} \) mol with a mean increase in perfusion of 44.7 ± 7.9% (Fig. 1). The maximal constrictor response was observed at \( 10^{-8} \) mol, significantly decreasing perfusion by −52.1 ± 11.1%. Following ACL transection, the dilatory phase of the SP response curve was effectively eliminated (Fig. 5). We did not measure systemic effects associated with the application of SP to the joint.
vasculature; however, blood flow did return to baseline levels between doses, indicating that responses to SP were probably not affected by systemic blood pressure changes.

To test the dilatory responsiveness of vascular smooth muscle, sodium nitroprusside was administered to both the resting vasculature and phenylephrine-precontracted vessels. Nitroprusside increased perfusion in the resting vasculature, but it was more effective in phenylephrine-precontracted vessels in both control and ACL-deficient knees with no significant differences between the two groups.

PGE₂ increased perfusion in control knees in a biphasic manner (Fig. 6). This response was maximally observed at $10^{-12}$ and $10^{-8}$ mol, increasing perfusion by 44.4 ± 9.3 and 42.6 ± 9.1%, respectively. ACL deficiency led to alterations in the dose-response curve compared with that observed in control knees. At concentrations ranging from $10^{-14}$ to $10^{-11}$ mol in the ACL-deficient knee, PGE₂ induced a significant decrease in perfusion. Higher concentrations of PGE₂ were still able to induce an increase in perfusion, although this response was still significantly lower than that observed in the control knee.
Reactive hyperemia assessment. Reactive hyperemia was assessed by femoral artery occlusion and reperfusion in both ACL-deficient and control knees. Femoral artery occlusion resulted in a $-73.6 \pm 7.3\%$ decrease in perfusion in control knees, and a $-73.0 \pm 5.8\%$ decrease in ACL-deficient knees ($P = 0.95$). Release of the femoral artery clamp was followed by reperfusion hyperemia of $74.3 \pm 11.1\%$ in control knees but of only $25.8 \pm 4.4\%$ in ACL-deficient knees (Fig. 7).

**DISCUSSION**

This study examined the effects of vasoactive mediators in the ACL-deficient model of OA, using high-resolution LSPI to detect rapid changes in blood flow (17, 18). Following 6 wk of ACL deficiency, the responsiveness of the MCL vasculature was assessed, baseline blood flow in the MCL showed significant 2.5-fold increases, and vasodilatory responses to ACh,
bradykinin, SP, histamine, and low doses of PGE2 were abolished. The response to shear stress was also abrogated in the ACL-deficient knee. Although no specific means of assessing receptor function or modes of action were employed and remain speculative, the physiological responses examined highlight the pathological condition of the vasculature in the ACL-deficient knee, a tissue that has not been extensively studied in OA.

Ligament rupture is a complex injury with multiple phases of healing. Ligament healing generally follows an orderly process of hemorrhage, inflammation, proliferation, and remodeling (19, 55). Unfortunately, partial ACL injuries show limited healing, whereas complete rupture effectively exhibits no healing response (24). The resultant chronic joint instability induces structural and physiological changes in intact supporting joint structures such as the MCL, which exhibits changes in mechanical properties and blood flow similar to those seen during scar formation after a direct injury (6, 9, 41).

Chronic joint instability often culminates in OA, with ACL deficiency in rabbits following a definable time line. At the 6-wk time point, we observed synovial hyperplasia, capsular thickening, MCL scarring, bucket handle medial meniscal tears, and increased knee joint diameter. There was no cartilage fibrillation or osteophyte formation. Similar results have been reported for ACL-deficient rabbit knees by other groups (2, 25, 31, 44, 57), where joint degeneration and inflammation occurred during the first 8 wk, and both degenerative and regenerative processes occur after 8 wk (44). Molecular alterations have been observed in the ACL-transected knee 6 wk postsurgery (26) and indicate that early-stage OA is present 6 wk after ACL transection.

Fig. 7. Effects of femoral artery occlusion and reperfusion hyperemia in the ACL-deficient knee and control knees. Femoral arteries of ACL-deficient and control limbs were occluded for 4 min, and perfusion change was measured in the MCL. Following occlusion, reperfusion hyperemia was also measured in the MCL. Values are means ± SE given as %change. *Significant difference between control and ACL-deficient knees, P < 0.05. (Student’s t-test).

Traditional evaluation of endothelial function involves use of physical stimuli, such as shear stress; intraluminal use of various vasodilators, such as ACh; and vascular measurement (27). Endothelial dysfunction results from a decrease in the bioavailability of NO due to both decreased formation and enhanced degradation (27). Excessive generation of reactive oxygen species, as has been found to occur in many forms of arthritis, may contribute to decreased NO-generating capacity. Because stimulation of vascular smooth muscle by NO results in smooth muscle relaxation, decreased bioavailability and production capacity of NO would impair normal vascular smooth muscle relaxation (27). In the present study, we chose to use a variety of dilatory stimuli that function through diverse mechanisms of action to establish a broad evaluation of both endothelial function and vascular reactivity. Topical administration of these vasodilators was used to minimize systemic effects on vascular tone and cardiac output. Because topical administration of these agents to an exposed vascular bed elicited a traditional dilatory response that was reproducible in the control vasculature, topical administration was considered an acceptable means of drug delivery.
Ach, bradykinin, and histamine induce endothelium-dependent relaxation of vascular smooth muscle through activation of eNOS and NO. Ach is known to induce dilation in vascular smooth muscle via the M2 and M3 muscarinic receptors located on endothelial cells, and it has become a diagnostic tool for determining endothelial function (10, 37, 38, 42). Bradykinin, via the B2 receptor, increases intracellular calcium in both vascular smooth muscle cells and endothelial cells (3, 4, 22, 23). Histamine also elicits endothelium-dependent relaxations of conduit elastic and muscular peripheral blood vessels such as the aorta, common carotid, femoral artery, and superior mesenteric arteries through activation of histamine H1 receptors (12, 13, 30). In a study of the canine lingual artery, removal of the endothelium drastically reduced the dilatory effects of histamine (13). ACL deficiency led to an abrogation of normal responses to Ach, bradykinin, and histamine, likely indicating the presence of a dysfunctional endothelium in this model of OA.

Previous reports illustrate the necessity of an intact endothelium for SP-dependent vascular relaxation (45). Use of the NO synthase inhibitor N\textsuperscript{G}-nitro-L-arginine in the rabbit isolated jugular vein showed that SP-induced relaxation is mediated by the production of NO and that removal of the endothelium eliminates this dilatory response. These authors have also reported that at higher concentrations (≥100 nM), SP elicits a contractile response (45). The present results show that similar responses to SP occur in the normal vasculature supplying the MCL, although the contractile effect occurred at a slightly higher concentration. In rabbit coronary arteries, SP has been shown to induce endothelium-dependent relaxation through the production of NO (45). In these same tissues, SP has been shown to induce endothelium-dependent contraction through the production of thromboxane A\textsubscript{2} (TxA\textsubscript{2}), which may be inhibited by addition of the cyclooxygenase inhibitor aspirin, the TxA\textsubscript{2} antagonist ONO-3708, and the TxA\textsubscript{2} synthetase inhibitor OKY-046. Therefore, the vasoactive effects of SP are likely dependent on the production of several other mediators, and the loss of the dilatory phase of the SP response in ACL-deficient knees may indicate a decreased capacity of the vasculature to produce or respond to NO. The decreased contractile response observed in the rabbit ACL-deficient pathology may result from alterations in SP endothelial signaling cascades, decreased capacity to produce TxA\textsubscript{2}, or some other mechanism(s). Because SP effects are both tissue and species specific, alterations observed in the rabbit ACL-deficient model may not be applicable to human OA, and this warrants further investigation (34).

PGE\textsubscript{2} differs from the other mediators studied in that this molecule acts directly on vascular smooth muscle. The EP2 and EP4 receptors are responsible for mediating the effects of PGE\textsubscript{2} on vascular smooth muscle and are solely responsible for the vasodilatory effects of PGE\textsubscript{2} (15, 32). Activation of these receptors increases cytosolic levels of cAMP. In the present study, PGE\textsubscript{2} elicited a biphasic response in the normal cohort (Fig. 6). A biphasic response was observed in the ACL-deficient cohort, with PGE\textsubscript{2} causing a decrease in perfusion at lower concentrations and an increase in perfusion at higher concentrations, although this response was diminished compared with controls. These results likely indicate the presence of two receptors for PGE\textsubscript{2}, each with different affinities for the endogenous ligand; the higher affinity receptor is probably altered or absent in the ACL-deficient knee. Because the EP4 receptor has a higher affinity for PGE\textsubscript{2} than the EP2 receptor (16), the EP4 receptor is most likely altered in the ACL-deficient cohort. The lack of a vasodilatory response at low PGE\textsubscript{2} concentrations in the ACL-deficient cohort may reflect a downregulation or reduced sensitivity of this receptor.

Flow-mediated dilation has become a valuable tool to detect endothelial dysfunction because of the noninvasive nature of this diagnostic method (27, 52, 54). Stretch-activated receptors induce NO production in endothelial cells via eNOS and result in smooth muscle relaxation (47). Femoral artery occlusion resulted in similar decreases in perfusion in both control and ACL-deficient knees, indicating that the vasculature in the ACL-deficient knee has similar capacity for perfusion changes. However, reperfusion following femoral artery occlusion only resulted in a 25% increase in perfusion in ACL-deficient knees compared with an 84% increase in perfusion detected in control knees. This diminished dilatory response in the vasculature indicates profound endothelial dysfunction in this model of OA. Dysfunctional endothelial cells produce proinflammatory mediators and matrix-degrading proteins, increase collagen synthesis, and increase inflammatory cell migration, and they may play a role in joint degeneration in this OA model (11, 40, 49, 53).

Rupture of the ACL and induction of OA initiates widespread effects that alter the normal homeostatic mechanisms of other supporting structures in the knee, including the MCL (6). Vascular inflammation and endothelial dysfunction are pathologies that not only affect the vasculature but also are responsible for infiltration of leukocytes into the surrounding structures, and they may be involved in initiating inflammatory responses from resident cells in various tissues (reviewed in Ref. 40). In combination with the altered mechanical environment in the ACL-deficient knee, endothelial dysfunction and vascular inflammation may provide a link between inflammation observed in the joint space and adaptive changes occurring in supporting structures. Amelioration of endothelial and vascular dysfunction not only may provide new insights into OA progression but also may present new methods to initiate joint repair by reducing joint inflammation and associated adaptive changes that may contribute to progression of the disease.

In conclusion, ACL deficiency markedly decreases the responsiveness of the MCL vasculature. The dilatory response to Ach, bradykinin, histamine, and SP and one phase of the PGE\textsubscript{2} dilatory response were abolished. Response to shear stress was also attenuated in the ACL-deficient rabbit knee. Instead, abnormal contractive responses were observed with lower concentrations of PGE\textsubscript{2} and with administration of Ach, bradykinin, histamine, and SP. Loss of these responses indicates decreased vascular reactivity and endothelial dysfunction in ACL-deficient knees. Further understanding of adaptive mechanisms and vascular changes in degradative OA may provide new therapeutic avenues for slowing progression of the disease and initiating repair processes.

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