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Characterization of sulfate, proline, and glucose transport systems in anterior cruciate and medial collateral ligament cells

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Abstract: The present study was undertaken to define the nature of key transport processes for sodium, glucose, proline, and sulfate in primary culture of canine anterior cruciate ligament (ACL) and medial collateral ligament (MCL) cells. Uptake studies using radiolabeled isotopes were performed and Na,K-ATPase activity was determined in cell lysates. At 25 °C both ACL and MCL cells showed a significant uptake of ^{86}Rb. Ouabain inhibited Rb uptake by 55% in ACL cells and by 60% in MCL cells. The transport activity of Na,K-ATPase in intact cells was calculated to be 57 and 71 nmol·(mg protein)$^{-1}$·(15 min)$^{-1}$ respectively. The enzymatic activity of Na,K-ATPase in cell lysates was observed to be 104 for ACL cells and 121 nmol·(mg protein)$^{-1}$·(15 min)$^{-1}$ for MCL cells. Cytochalasin B, a known inhibitor of sodium-independent D-glucose transport, completely inhibited D-glucose uptake in ACL and MCL cells. Removal of Na^+ or addition of 10^{-5} mol/L phlorizin, a potent inhibitor of the sodium-D-glucose cotransporter, did not alter D-glucose uptake, suggesting that glucose entered the cells using a sodium-independent pathway. Both ACL and MCL cells exhibited high sulfate uptake that was not altered by replacement of Na^+ by *N*-methyl-D-glucamine, whereas DIDS, an inhibitor of sulfate/anion exchange abolished sulfate uptake in both cell types. Thus, neither cell type seems to possess a sodium-sulfate cotransport system. Rather, sulfate uptake appeared to be mediated by sulfate/anion exchange. Proline was rapidly taken up by ACL and MCL cells and its uptake was reduced by 85% when Na^+ was replaced by *N*-methyl-D-glucamine, indicating that proline entered the cells via sodium-dependent cotransport systems. The data demonstrate that both ACL and MCL cells possess a highly active sodium pump, a secondary active sodium-proline cotransport system, and sodium-independent transport systems for D-glucose and sulfate.

Key words: ligament, fibroblasts, transport, proline, sulfate, glucose, sodium.

Résumé : La présente étude a eu pour but de définir la nature des principaux processus de transport du sodium, du glucose, de la proline et du sulfate dans des cellules du ligament croisé antérieur (LCA) et du ligament collatéral interne (LCI) du genou canin en culture primaire. On a effectué des études de captation à l'aide d'isotopes radiomarqués, et déterminé l'activité Na,K-ATPase dans les lysats de cellules. À 25 °C, tant les cellules du LCA que du LCI ont montré une captation significative du ^{86}Rb. L'ouabaïne a inhibé la captation du Rb de 55 % dans les cellules du LCA et de 60 % dans les cellules du LCI. L'activité de transport de la Na,K-ATPase dans les cellules intactes a été de 57 et de 71 nmoles·(mg de protéine)$^{-1}$·(15 min)$^{-1}$, respectivement. L'activité enzymatique de la Na,K-ATPase dans les lysats a été de 104 nmoles·(mg protéine)$^{-1}$·(15 min)$^{-1}$ pour les cellules du LCA et de 121 pour les cellules du LCI. La cytochalasine B, un inhibiteur bien connu du transport de D-glucose indépendant du sodium, a complètement inhibé la captation du D-glucose dans les cellules du LCA et du LCI. Le retrait du Na^+ ou l'addition de 10^{-5} mol/L de phlorizine, un puissant inhibiteur du cotransporteur sodium-D-glucose, n'ont pas modifié la captation du D-glucose, ce qui laisse croire que le glucose est entré dans les cellules par une voie indépendante du sodium. Tant les cellules du LCA que du LCI on montré une captation élevée du sulfate qui n'a pas été modifiée en substituant la *N*-méthyl-D-glucamine au Na^+, alors que le DIDS, un inhibiteur de l'échange sulfate/anion a supprimé la captation du sulfate dans les 2 types de cellules. Ainsi, aucun des 2 types de cellules ne posséderait un système de cotransport sodium-sulfate, la captation de sulfate semblant plutôt être véhiculée par l'échange sulfate/anion. La proline a été rapidement capturée par les cellules du LCA et du LCI, et sa captation a été réduite de 85 % lorsque la *N*-méthyl-D-glucamine a été substituée au Na^+, ce qui indique la proline est entrée dans les cellules par des systèmes de cotransport dépendants du sodium. Les résultats démontrent que les cellules du LCA et du LCI possèdent une pompe à sodium très active, un système de cotransport sodium-proline actif secondaire, et des systèmes de transport du D-glucose et du sulfate indépendants du sodium.

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Mots clés : ligament, fibroblaste, transport, proline, sulfate, glucose, sodium.

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Introduction

The human knee and the equivalent stifle joint of mammals are complex joints composed of the articulating surfaces of femur and tibia. This noncongruent joint is stabilized by 2 intraarticular ligaments, the anterior and posterior cruciates; 2 extraarticular ligaments, the medial and lateral collateral ligaments; as well as cartilage and meniscus that provide lubrication and load distribution. Mechanical load is critical in maintenance of structure and function of ligaments and its effects are mediated through regulation of matrix turnover (Wiig et al. 1991). The uptake of ions, energy sources, and precursors for collagen and proteoglycans is an important in regulation of matrix turnover, cell metabolism, and cellular ion homeostasis. We have thus characterized the transport processes involved in sodium, glucose, proline, and sulphate transport in primary cultures of ACL and MCL cells.

Studies in vivo and in vitro have revealed marked differences in the healing abilities of the anterior cruciate ligament (ACL) and the medial collateral ligament (MCL) (Arnold et al. 1979; Cabaud et al. 1979; Nagineni et al. 1992; O'Donoghue 1955; O'Donoghue et al. 1971). In vivo, the medial collateral ligament heals with rare exception. The resultant ligament scar is able to withstand physiologic load and rarely requires operative repair or reconstruction. This situation can be contrasted sharply with the anterior cruciate ligament in which failure of the repair process is quite common (Arnold et al. 1979; Cabaud et al. 1979; O'Donoghue 1955; O'Donoghue et al. 1971). Complete ACL injuries do not heal in vivo and controversy exists as to the ability of the ACL to recover structural and functional integrity after a partial injury. In vitro this difference is also observed in wound closure assays of confluent primary cultures in which ACL fibroblasts show a markedly lower proliferation and migration potential when compared with MCL fibroblasts (Nagineni et al. 1992). These studies were undertaken to determine if the observed differences between ACL and MCL in vivo and in vitro could in part result from differences in the transport systems for sodium, sulfate, glucose, and proline.

Materials and methods

Isolation of canine knee ligament fibroblasts

Isolation of canine knee ligament fibroblasts was performed as described by Nagineni et al. (1992) and Ross et al. (1990) with several modifications. Canine knee ligaments were obtained from male animals 14 –24 months that were sacrificed for other studies at the Hospital of Special Surgery. ACL and MCLs were harvested under aseptic conditions and placed in sterile medium M199 containing 10% antibiotic-antimycotic solution (Gibco-BRL, Grand Island, N.Y.). A portion of the ligaments was trimmed at the tibial and femoral ends and discarded. The outer synovial layer was removed via sharp dissection and the ligaments were finely minced into 20–40 pieces and placed into T-75 flasks (Becton Dickinson, Franklin Lakes, N.J.) containing medium M199 with 10% fetal calf serum (Gibco-BRL) and 1% antibiotic–antimycotic solution. The flasks were incubated at 37 °C in a humidified atmosphere of 5% CO_2 : 95% air. The cells migrated out of the explants, attached to the flasks, and divided. The explanted pieces were removed after 10–12 days or when a sufficient number of cell colonies had formed. A confluent monolayer was routinely achieved in 2 weeks. Serial passages were performed as follows: Cells were washed with M199 and detached from the flasks by treatment for 2 min with 0.25% trypsin – 1 mmol/L EDTA in Ca^{2+}- and Mg^{2+}-free Hanks Balanced Salt Solution (Gibco-BRL) at 37 °C. The suspension was subsequently diluted with the growth medium, cells were collected by centrifugation (3500*g* for 10 min), suspended in the growth medium and seeded at a density of 1×10^5 cells/mL. Passages were limited to a maximum of 4.

Uptake experiments

ACL and MCL cells were grown to near confluency in 6-well culture plates (3×10^5 cells/well). For determination of uptake of radiolabeled rubidium, glucose, sulfate, and proline, the culture medium was removed and the cells were incubated for 6 min with a transport medium that contained (in mmol/L): 135 NaCl, 5 RbCl, 0.75 $CaCl^2$, 0.75 $MgCl^2$, 5 D-glucose, and 15 *N*-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), adjusted with Tris to pH 7.4. Differences in the incubation media are indicated in the figure legends. Incubation took place for 15 min at room temperature. At 30 min there was slight loss of linearity of solute uptake therefore an incubation period of 15 min was selected. Transport was terminated by rapid removal of the transport medium. The cells were rinsed 3 times with ice-cold phosphate buffered saline, pH 7.4, and solubilized in 2% sodium dodecyl sulfate containing 2 mmol/L ethylenediaminetetraacetate. Aliquots were mixed with scintillation fluid and analyzed for the amount of radioactivity taken up by the cells by standard liquid scintillation techniques (Deppe et al. 1997) Protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard. ^{86}Rb (specific activity 1–3 mCi/mg), 3H-D-glucose (uniformly labeled, specific activity 30 Ci/mmol), sulfate (carrier free), and 3H-L-proline (specific activity 50 Ci/mmol) were purchased from NEN-Life Science Products, Boston, Mass.

Na,K-ATPase assay

Cells were scraped from 6-well plates, rinsed, and suspended in ST buffer (250 mmol/L sucrose, 10 mmol/L triethanolamine, pH adjusted with HCl to 7.5). The cell suspension was flash-frozen in a dry ice–ethanol mixture and subsequently thawed at 37 °C. This freezing and thawing was repeated 6 times. All cells appeared to be lysed when the suspension was examined in the microscope. Cell lysates were incubated in presence or absence of 2 mmol/L ouabain in an incubation medium containing (in mmol/L) 50 imidazole, 6 Mg_2SO_4, 20 KCl, 100 NaCl, and 3 Tris-ATP, pH adjusted with HCl to 7.6 for 30 min at 37 °C. Appropriate blanks with ATP alone or cell lysate alone were included. The enzyme reaction was

Fig. 1. Rubidium uptake into ACL and MCL cells in primary culture. Ouabain, the specific inhibitor of Na,K-ATPase, was used to determine the activity of the active sodium pump in intact cells. Mean values are ± SE. Three different cell preparations were used for the experiments. Each experiment was performed in duplicate. TU, total uptake; +Ou, uptake in presence of 2 mmol/L ouabain; OuS, ouabain-sensitive uptake.

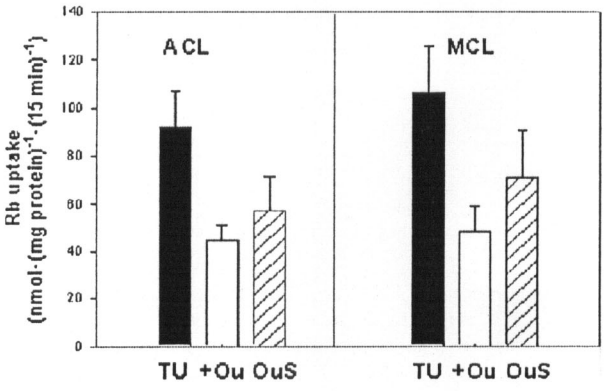


Table 1. Na,K-ATPase activity in homogenates of ACL and MCL cells.

	ATPase activity (nmol·(mg protein)$^{-1}$·(15 min)$^{-1}$)		
Cell type	Total ATPase	+ Ouabain	Na,K-ATPase
ACL	251±36	147±23	104
MCL	301±44	180±31	121

Note: Each experiment was done in duplicate (n = 4). Mean values ± SE are given. ACL, anterior cruciate ligament; MCL, median cruciate ligament.

stopped by addition of equal volume of ice-cold 10% trichloroacetic acid. After centrifugation (3500g/10 min) the inorganic phosphate liberated from the substrate ATP was determined as described earlier using ammonium molybdate in sulphuric acid and ascorbic acid (Kinne et al. 1971). The difference in inorganic phosphate released between samples incubated with and without ouabain is referred to as the Na,K-ATPase activity. The activity is expressed per 15 min so that it can be compared with the activity obtained from ^{86}Rb uptake experiments.

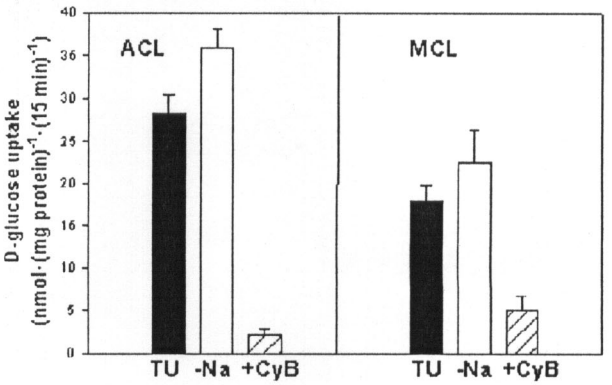
Results

Na,K-ATPase activity of ACL and MCL cells

One of the major plasma membrane transport systems involved in maintaining homeostasis of intracellular ions and the asymmetrical distribution of sodium and potassium across the plasma membrane is Na,K-ATPase. The activity of this transport system was investigated by the followinhg 2 approaches: (*i*) measurement of ouabain-inhibitable rubidium uptake into intact cells and (*ii*) determination of the sodium- and potassium-stimulated, ouabain-inhibitable ATPase activity in cell lysates.

In Fig. 1, the results of ^{86}Rb uptake studies are compiled in which rubidium was used as substitute for potassium.

Fig. 2. D-Glucose uptake into ACL and MCL cells in primary culture. Sodium-free solutions (replacement by *N*-methyl-D-glucamine) and cytochalasin B were employed to distinguish between sodium-dependent glucose cotransport systems and sodium-independent glucose transporters. Mean values are ± SE. Three different cell preparations were used for the experiments and each experiment was performed in duplicate. TU, total uptake; -Na, uptake in absence of sodium; +CyB, uptake in presence of 10^{-5} mol/L cytochalasin B.



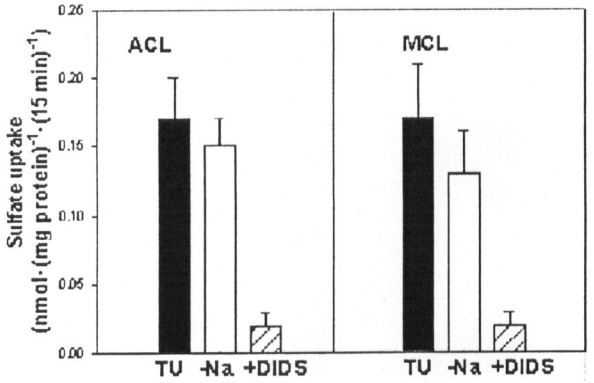
Both ACL and MCL show a significant uptake of rubidium at 25 °C. This uptake is reduced in both cell types by 87.4% ± 6% when the incubation temperature is lowered to 4 °C, suggesting that active transport of rubidium across the plasma membrane occurs. Rubidium uptake was ouabain sensitive in both cell types. In ACL cells, the inhibition was observed to be 54.8% ± 4.5%, whereas in MCL cells it was 60.2% ± 4.8%. Since the ouabain concentration used is known to inhibit canine Na,K-ATPase completely (Xie et al. 1996) a transport activity of 57.4 ± 14.1 nmol·(mg protein)$^{-1}$·(15 min)$^{-1}$ for ACL and 71.0 ± 16.2 nmol·(mg protein)$^{-1}$·(15 min)$^{-1}$ can be calculated for intact cells (Table 1).

The enzyme activity was also determined in cell lysates (Table 1). In ACL and MCL cells a significant ATPase activity can be observed, which is inhibited by ouabain 41.4% and 40.2%, respectively. MCL cells exhibited a slightly higher total ATPase and Na,K-ATPase activity compared to ACL cells in each experiment; however the difference was not statistically significant.

D-Glucose uptake by ACL and MCL cells

D-Glucose is the major metabolic substrate for fibroblasts and an important precursor for the synthesis of proteoglycans. The mode by which this sugar is taken up was studied. Both ACL and MCL fibroblasts avidly take up D-glucose at a similar rate (Fig. 2). This uptake is strongly temperature sensitive; at 4 °C only 5% ± 3% of the uptake at 25 °C is observed. Removal of sodium has no signifcant effect on D-glucose uptake. Similarly, addition of ouabain has only a slight inhibitory effect in ACL cells (20.8% ± 7%) and no effect on MCL cells. The effect of 5 × 10–5 mol/L phlorizin, a potent inhibitor of the sodium-D-glucose cotransporter was evaluated in 2 experiments and it also did not alter D-glucose uptake. In contrast, cytochalasin B, a known inhibitor of sodium-independent D-glucose transport almost completely abolished D-glucose transport (Fig. 2), suggesting that the

Fig. 3. Sulfate uptake into ACL and MCL cells in primary culture. Sodium-free solutions (replacement by *N*-methyl-D-glucamine) and 0.5 mmol/L DIDS (4,4′-diisiothiocyano-2,2′-disulfonic acid) were employed to distinguish between sodium-sulfate cotransport systems and sulfate/anion exchangers. Mean values are ± SE. Three different cell preparations were used for the experiments and each experiment was performed in duplicate. Sulfate concentration in the uptake medium amounted to 0.1 mmol/L. TU, total uptake; -Na, uptake in absence of sodium; + DIDS, uptake in presence of DIDS.



major fraction of D-glucose enters these cells via a sodium-independent pathway.

Sulfate uptake by ACL and MCL cells

Sulfate uptake was investigated because sulfate is a major component of proteoglycan synthesized by ligament cells. As demonstrated in Fig. 3, ACL and MCL cells take up sulfate to a similar extent. Sulfate uptake is decreased by 79.8% when the incubation temperature is lowered from 25 °C to 4 °C. Sulfate uptake in ACL and MCL cells is not significantly altered when sodium is removed from the incubation medium. However, 0.5 mmol/L DIDS completely abolishes sulfate uptake in ACL and MCL cells to the level observed at 4 °C. Thus, sulfate uptake in both cell types appears to be mediated by a sulfate/anion exchange system (Bissig et al. 1994; Elgavish and Meezan 1989) and not by a sodium-sulfate cotransporter (Bissig et al. 1994; Markovich et al. 1993).

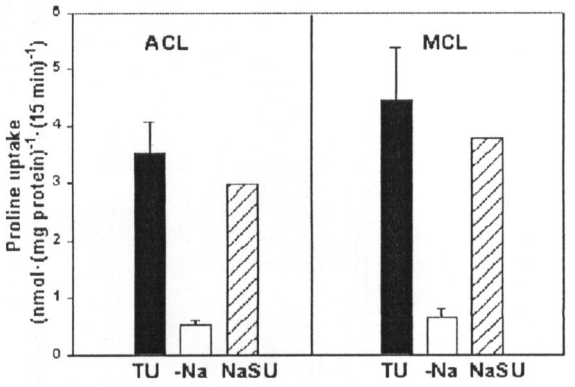
Proline uptake by ACL and MCL cells

Proline is an important precursor in collagen synthesis and therefore should provide a useful indicator for cell-specific function (Finerman et al. 1967) Proline is rapidly taken up by ACL and MCL cells (Fig. 4) This process is temperature dependent, with a 95% reduction in proline uptake seen at 4 °C. When sodium is replaced by *N*-methyl-D-glucamine the uptake of proline is decreased by 85% both in ACL and MCL cells, indicating that the majority of proline enters the cells via sodium-dependent cotransport systems.

Discussion

We have demonstrated the presence of Na,K-ATPase, sodium-independent D-glucose and sulfate transport, and sodium-dependent proline transport in ACL and MCL cells.

Fig. 4. L-Proline uptake into ACL and MCL cells in primary culture. Sodium-free solutions (replacement by *N*-methyl-D-glucamine) were employed to estimate the importance of sodium-proline cotransport systems for the overall uptake. Mean values are ± SE. Three different cell preparations were used for the experiments and each experiment was performed in duplicate. Proline concentration in the uptake medium amounted to 0.1 mmol/L. TU, total uptake; -Na, uptake in absence of sodium; NaSU, sodium-sensitive uptake.



The described transport pathways are active in both cell types in monolayer culture and no significant differences exist between ACL and MCL fibroblasts.

To our knowledge, this report is the first to measure Na,K-ATPase activity and to define sodium, glucose, sulfate, and proline transport in ACL and MCL cells. The transport studies employed in this paper were performed in cells after removal of the adjacent connective tissue to permit accurate characterization of the transport system. In vivo the surrounding connective tissue represents a large reservoir of unstirred fluid preventing reliable exchange between extracellular space and incubation medium and preventing reliable measurement of membrane transport systems. Tracer remaining in the extracellular space would also blunt any cellular signal because of the large differences between intra- and extracellular space.

Na,K-ATPase

The pump activity determined in the intact cell corresponds to ~100 nmol rubidium ions pumped per mg protein/15 min. If one assumes that 2 rubidium ions are translocated per ATP hydrolysed, an ATP hydrolysis of ~50 nmol per mg protein in 15 min can be estimated. In the cell lysate an ATPase activity of 100–125 nmol·(mg protein)$^{-1}$ ·(15 min)$^{-1}$ was observed. Thus, the activity determined in the cell lysate under optimum substrate and activator concentrations is about 2-fold higher than the transport activity measured in the intact cell. The lower activity found in the intact cell can, as in other cells, be explained by the low intracellular sodium concentration under physiological conditions and by the usual feedback on the pump exerted by the sodium and potassium conductances in the membrane. The higher temperature used in the enzyme assays has to be taken into account and may be in part responsible for the discrepancy between the measured ATPase activity and rubidium transport into intact cells.

The rate of ouabain-sensitive ^{86}Rb uptake observed in the current study compares well with values found in other fibroblasts, including 3T3 cells and human fibroblasts (Banerjee and Bosmann 1976; Brodsky 1990; Mendoza and Rozengurt 1987; Rozengurt and Heppel 1975; Russo et al. 1990). The inhibition of total ^{86}Rb uptake by the glycoside is quite similar (Mendoza and Rozengurt 1987) to that observed in these cells. The uptake of ^{86}Rb uptake observed in the present studies (~110 nmol·(mg protein)$^{-1}$ ·(15 min)$^{-1}$) was half of that observed with human hepatoma cell line HepG2 (~220 nmol·(mg protein)$^{-1}$ ·(15 min)$^{-1}$) (Wehner et al. 2002).

Glucose transport

Glucose transport by ACL and MCL cells exhibited characteristics typical for glucose uptake in other mammalian cells and fibroblasts (Kitagawa et al. 1989; MacKay et al. 1983). Of particular importance are the sensitivity to cytochalasin B and the insensitivity to sodium. This places the transport protein into the GLUT family, probably representing GLUT1, an assumption that has to be further substantiated. It is interesting to note that inhibition of the primary-active sodium pump by addition of ouabain (data not shown) inhibit D-glucose uptake only to about 20%. Since energy production by anaerobic glycolysis and glucose uptake are usually tightly coupled (MacKay et al. 1983), this finding might suggest that ion transport constitutes only a minor component of ATP-consuming cellular processes and (or) that aerobic metabolism of other substrate significantly contributes to ATP production in ACL and MCL cells. Compared to 3T3 cells stimulated with T3 (Romero et al. 2000) or with insulin (Bandyopadhyay et al. 1997) the rate of glucose uptake in ACL and MCL cells appears to be higher. However our studies were performed with 5 mmol/L D-glucose rather than 0.1mmol/L 2-Deoxy D-glucose used in these other studies. Thus a higher uptake rate would be expected in our studies.

Sulfate transport

The main characteristics of sulfate uptake into ACL and MCL cells are its sodium independence and the sensitivity to DIDS when low sulfate concentrations were used in the transport studies. The results closely resemble those obtained by Elgavish in human lung fibroblasts (Elgavish and Meezan 1989, 1991) where both a high-affinity, low-capacity DIDS-sensitive and a low-affinity transport system have been identified. The transport properties of ACL and MCL are consistent with a SO_4/X^- exchange mediating the translocation of sulfate across the membrane (Elgavish and Meezan 1989). The rate of sulfate transport in the ACL/MCL cells are quite to similar those observed with these human lung fibroblast cell lines, when corrected for the different sulfate concentrations used.

Proline uptake

Proline uptake into cultured human skin fibroblasts was first characterized in detail by Gazzola et al. (1980). These authors observed that proline uptake was almost completely sodium dependent with sodium independent uptake accounting for 8% of total uptake. This finding is corroborated in ligament fibroblasts by our study. The rate of proline uptake found in canine ACL and MCL is about 5 times lower than that observed in human skin fibroblasts. The studies performed in skin fibroblasts were performed at 37 °C, with proline-depleted fibroblasts (Gazzola et al. 1980). In this study, the ACL and MCL fibroblasts were not proline depleted and were studied at 25 °C. The proline uptake observed in the present studies (2 nmol·(mg protein)$^{-1}$ ·(15 min)$^{-1}$) with ACL/MCL cells is higher than observed with MDCK cells (0.5 nmol·(mg protein)$^{-1}$ ·(15 min)$^{-1}$) (Zelikovic and Wager-Miller 2001).

Comparison between transport properties of ACL and MCL cells in primary culture

Despite differences between the healing capability of ACL and MCL observed in vivo, the transport of metabolites and uptake of proline and sulfate, precursors for extracellular matrix synthesis (Finerman et al. 1967; Phang et al. 1971; Zafra et al. 1994) are similar for the 2 cell types. It is possible that specific characteristics of the cells may be lost in primary culture in vitro; however this is unlikely since differences in proliferation, actin organization, expression of procollagen mRNA, and signal pathways for adhesion to integrin have been demonstrated in primary culture ACL and MCL cells (AbiEzzi et al. 1995; Nagineni et al. 1992; Sung et al. 1996*a*, 1996*b*; Wiig et al. 1991). In addition, the cells used in the current study demonstrated the classic differences in morphology that have previously been described for primary ligament cell culture (Lyon et al. 1991) with MCL cells forming a tight network of swirling cells, whereas ACL cells were arranged in parallel strips or bands.

Although no significant differences in the activity of transport systems investigated in the present study were observed between ACL and MCL cells, it is possible that other transport systems involved in ion homeostasis (e.g., those involved in pH or volume regulation), the presence of transport regulators (Fénéant-Thibault et al. 1991; Kudo et al. 1996) or different velocities of cell specific transport systems might contribute to the differences noted in the healing response in ACL and MCL fibroblasts in vitro and in vivo. It should also be noted that these systems were studied in the absence of applied mechanical load. It remains to be determined whether these or other transport systems in ligament fibroblasts are sensitive to mechanical stress. Furthermore the matrix composition, duration, and magnitude of mechanical load experienced by ACL and MCL cells could be different in vivo, leading to differences in the transport properties in vivo.

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