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Differential effects of embryonic immobilization on the development of fibrocartilaginous skeletal elements

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Abstract—The importance of mechanical influences during skeletal development has been well established in both experimental studies and computer models. Under conditions of embryonic immobilization, it has been observed that the early stages of joint formation proceed normally (up to and including interzone formation), but the later stages of joint cavitation and maintenance are impaired, resulting in fusion of the cartilaginous elements across the presumptive joint line. Two structures in particular are noticeably absent from late-stage synovial joints in immobilized chick embryos: the menisci of the tibiofemoral joint and the plantar tarsal sesamoid of the tibiotarsal joint. Both of these fibrocartilaginous structures are known to serve mechanical functions in postnatal animals, helping to distribute loads within the joint and, in the case of sesamoid structures, to provide a mechanical advantage to muscles acting across the joint. We demonstrate in this study that embryonic immobilization differentially affects the developmental fate of these two distinct fibrocartilages. The absence of the plantar tarsal sesamoid in late-stage immobilized embryos is due to a failure in the initial formation of this structure. In contrast, the early stages of meniscus formation proceed normally. Without the normal mechanical stimuli of skeletal muscle contractions, however, the meniscus fails to mature and ultimately degenerates.

Key words: *embryonic immobilization, fibrocartilage, joint development, mechanical loading, menisci, sesamoids.*

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INTRODUCTION

Normal synovial joint formation consists of two phases. First, the developing mesenchymal blastema differentiates into a cartilaginous model of the future long bone. Adjacent skeletal elements are separated by thin bands of mesenchymal cells known as interzones. Although the biology of the interzone is poorly understood, it is believed that these structures differentiate into three layers; two outer chondrogenic layers that will cover the cartilage anlage, and an intermediate layer that contributes to the formation of intra-articular structures such as ligaments, menisci, and the synovium (**Figure 1, (a), (b)**). Subsequent to the formation of the interzone is joint cavitation, the process by which adjacent cartilaginous elements separate to form two distinct articulating joint surfaces (**Figure 1, (c)**). Only if both of these developmental processes proceed undisturbed will normal formation and maintenance of synovial joints be observed.

Experimental investigations have demonstrated a very close relationship between the mechanical forces resulting from skeletal muscle contractions and the normal sequence of morphological events that occur during skeletogenesis (2). In particular, the development of the synovial joints has been shown to be especially sensitive to changes in the mechanical loading environment (3–6). Although anatomic and histologic descriptions of synovial joint formation differ slightly, depending on the degree of immobilization that is attained, the early stages

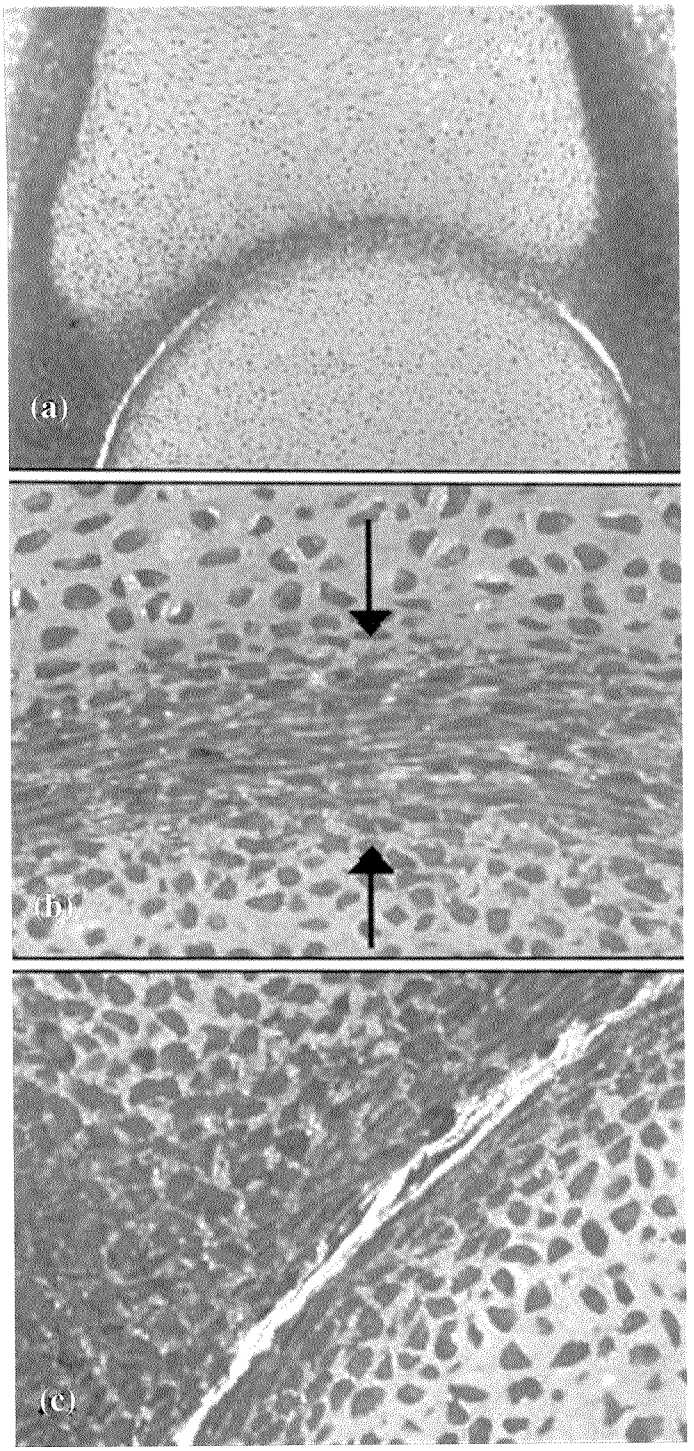

Figure 1.
(a) Day 13 proximal interphalangeal joint from a chick embryo. One-micron thick, Epon-embedded section stained with toluidine blue. (b) Central region of late-stage interzone enlarged from figure in (a). The interzone is demarcated by arrows above and below. (c) Early-stage cavitation depicting separation of two articulating cartilaginous elements enlarged from figure in (a).

of joint formation do not appear to be adversely affected by immobilization (i.e., up to and including formation of the interzone). However, the subsequent events of cavitation and maintenance of the articulating surfaces are dramatically altered, with lack of cavitation, partial cavitation, and postcavitational joint fusion occurring in the absence of skeletal muscle contractions (**Figure 2**). In contrast to the numerous immobilization studies that have been performed postnatally, only one documented study has been reported in which increased embryo motility was induced in developing chicks (7). Increased muscle activity led to temporary increases in the size of the joint cavity. To date, however, no studies have been performed in which sustained levels of increased loading have been maintained.

Because of the ease of manipulation, most experimental investigations into the role of mechanical factors in joint development have been performed using the chick embryo (3–6). The first involuntary muscle contractions in the developing chick occur on Day 3 postfertilization, although it is not until Day 7 that the distal segments of the limb begin to move, and only sometime between Days 8–10 that independent movement of the digits occurs (8). Embryonic movement increases in magnitude and frequency until approximately Day 17, after which there is a reduction as the chick prepares to hatch on Day 21.

Efforts to quantify embryonic movement have been largely confined to visual observations through the ``windowed'' eggshell for finite periods of time throughout the day. In our laboratory, we have taken the approach of measuring deformations of the eggshell resulting from motion of the embryo. Vibrations in the shell disrupt the magnetic field of a micromagnet attached to the shell, and these disruptions in the field can be detected using a Hall Effect Transducer (**Figure 3, (a)**). The signal from the transducer is amplified, filtered, and recorded with a computer, and movement patterns are subsequently analyzed. The device that we have developed is a variation on a circuit designed by Lighton and colleagues (9) for studies of abdominal muscle contractions in insects and is capable of detecting micron-level shell deflections. **Figure 3, (b)** demonstrates a 2-min trace recording from a Day-19 chick embryo documenting two distinct movements of the embryo within the egg. Detailed analyses of signal characteristics in conjunction with visual observations of the embryo using an arthroscope will allow us to discern signals caused by rolling of the entire embryo versus independent limb movements due to muscle contractions. Reliable methods of quantifying movement pat-

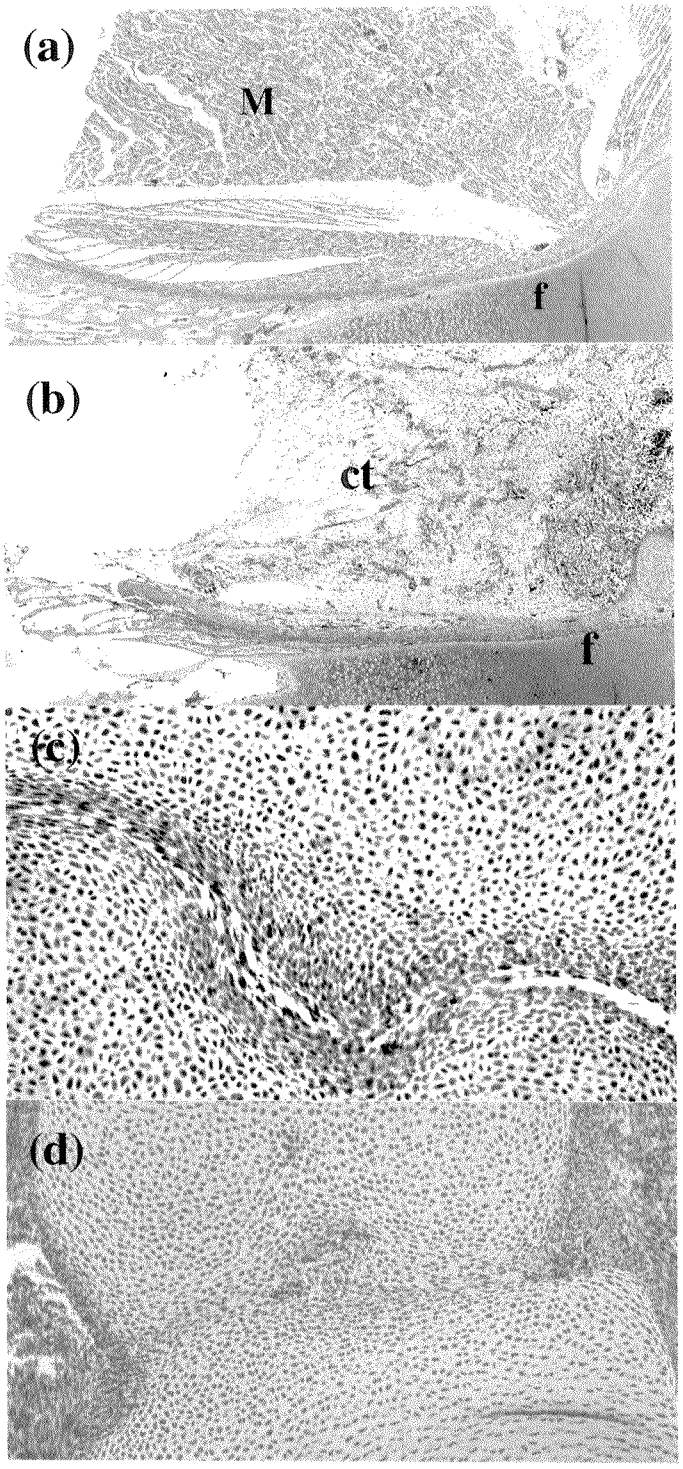

Figure 2.
Day 13 chick embryo control (a) and immobilized (b) hamstring muscle group and femur. (f=femur, M=muscle, ct=connective tissue). Day 13 distal interphalangeal joint from (c) control and (d) immobilized embryos. Note the fused cartilaginous elements in the immobilized joint compared to the fully cavitated control joint. (6-μm paraffin sections stained with fast green, hematoxylin, and safranin O).

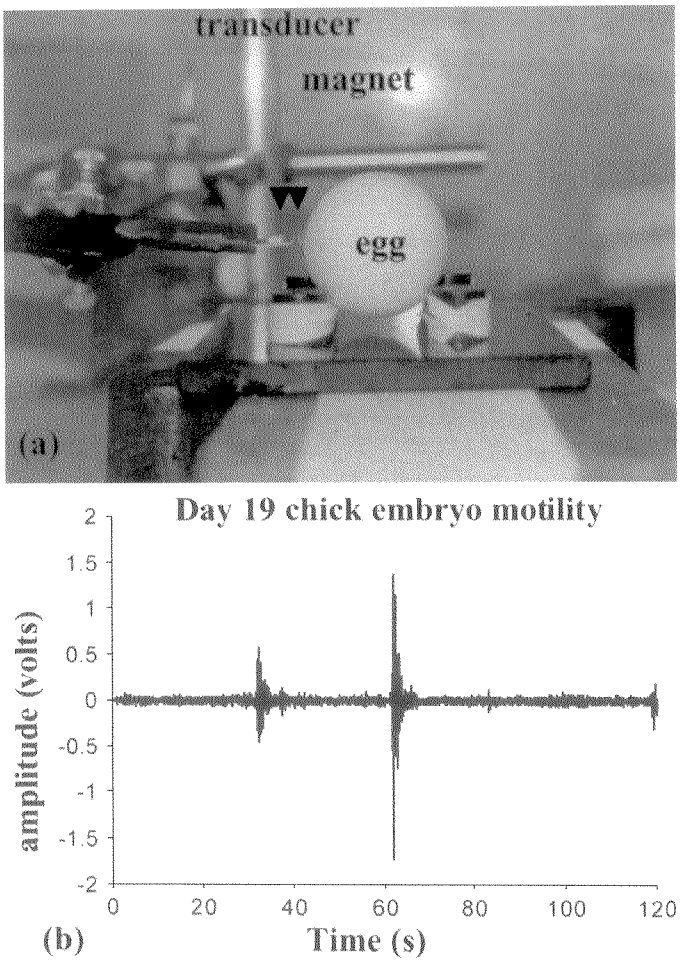

Figure 3.
(a) Set-up for detecting embryonic movements. Motion of the embryo against the eggshell results in vibrations of the shell. These vibrations disrupt the magnetic field of the magnet attached to the shell, and the variations in magnetic field are detected by a Hall Effect Transducer. (b) A 2-min trace recording from the Hall Effect Transducer signal of a Day 19 chick embryo. Two distinct motion events of the embryo are shown, with the start of a third event at the end of the trace.

terns in developing chick embryos are necessary in order to quantify the extent of any experimental perturbations in mechanical loading.

Four approaches have traditionally been employed for inducing embryonic immobilization in developing chick embryos. These include a) administration of neuromuscular blocking agents *in ovo* (3,5,6,10); b) *in vitro* organ culture of limbs (4); c) microsurgical ablation of the lumbosacral portion of the neural tube (11); and d) ablation of the somites themselves using UV irradiation. Each approach has its own advantages and disadvantages, but the technique of pharmacological intervention using

neuromuscular blocking agents is by far the most widely used because of its relative ease. The most common drug employed is decamethonium bromide (DMB) in single doses into the air sac (12,13), periodic application onto the chorioallantoic membrane (5,6,14), or infusion via the chorioallantoic blood vessels (3,10).

Although much attention has been focused on the effect of embryonic immobilization on overall synovial joint formation, relatively little is known about the role of mechanical influences in the formation of joint-associated structures such as the menisci, sesamoids, and synovium. In adults, sesamoids are bones that have formed via endochondral ossification of cartilages found within tendons. Prior to ossification, these structures can be considered fibrocartilaginous because of the cartilaginous nature of the sesamoid proper and the fibrous nature of the surrounding tendon. In late-stage synovial joints from immobilized chick embryos, the meniscus of the tibiofemoral joint and the plantar tarsal sesamoid of the tibiotarsal joint are conspicuously absent. Both of these structures are known to serve mechanical functions in postnatal animals, helping to distribute loads within the joint and, in the case of sesamoid structures, to provide a mechanical advantage to muscles acting across the joint. Although embryonic sesamoids and menisci are both fibrocartilaginous, it is possible that sesamoids may form as a result of tissue differentiation within regions of the tendon that experience high hydrostatic compressive stresses (15,16). The aim of this study was to investigate whether the absence of sesamoids and menisci in late-stage immobilized chick embryos is due to a failure in the initial formation of these elements, or, rather, to development of the structures followed by degeneration in the absence of normal functional skeletal muscle contractions. We demonstrate in this study that embryonic immobilization differentially affects the developmental fate of these two distinct fibrocartilages.

METHODS

Limb immobilization was pharmacologically induced in embryonic chicks starting at Day 6 of incubation using a well-established neuromuscular blocking agent, DMB (0.25 ml in 0.02 percent Hank's Buffered Saline Solution), dropped onto the chorioallantoic membrane twice daily, while control embryos received equal volumes of saline. Embryos were sacrificed at daily intervals from 8 to 17 days of incubation, and intact tibiotarsal and tibiofemoral joints were dissected free, fixed for 24 hours in 4 percent paraformaldehyde, decalcified in 0.4 M EDTA, and embedded in paraffin. Six-micron serial sections were cut and stained using routine histologic procedures with a trichrome stain of fast green, hematoxylin, and safranin O.

RESULTS

In control embryos, meniscal condensation was evident in the tibiofemoral joint by Day 8 of incubation (**Figure 4, (a), (c)**). Minimal staining of proteoglycans in the cartilage of the tibial and femoral anlagen was detected at this stage. By Day 9, the presumptive meniscus had obtained a more definite shape, with some decrease in cellularity in the tissue immediately surrounding the meniscus accompanying the early stages of cavitation of this structure (**Figure 4, (e)**). By Day 11 and beyond, concomitant with the onset of cavitation of the entire tibiofemoral joint, the meniscus was well formed and distinct from the tibia and femur, with a well-defined inner tapered region and well-vascularized outer region (**Figure 4, (g)**).

In the immobilized embryos, the early stages of meniscus formation proceeded normally, with a meniscal condensation evident by Day 8 (**Figure 4, (b), (d)**). By Day 10, however, the presumptive meniscus was infused with numerous brightly staining erythrocytes entering from the lateral margins of the tissue. These cells were associated with the degeneration and eventual disappearance of the meniscus by Days 11–12 of incubation (**Figure 4, (f)**), and were accompanied by fusion of the cartilaginous elements of the tibiofemoral joint. By Day 13 and beyond, loose fibrovascular connective tissue remained where the menisci once were located (**Figure 4, (h)**).

In contrast to the initial formation of the meniscus followed by subsequent degeneration under conditions of immobilization, the absence of the plantar tarsal sesamoid in late-stage immobilized embryos appeared to be due to a complete failure of initial formation. Before Days 10–11, the sesamoid of control embryos was evident as a diffuse condensation of cells in the approximate shape and location of the future sesamoid, with no positive staining for proteoglycans within the region itself at this stage (**Figure 5, (a)**). By Day 11, the sesamoid had fully formed as a separate entity articulating with the tibiotarsal joint (**Figure 5, (c)**). As development progressed, this structure enlarged and became more and more prominent.

In the immobilized embryos, even as early as Day 9, the tibiotarsal joint was abnormally ankylosed, with a marked reduction in the tissue space where the sesamoid would be expected to form between the tibiotarsal joint and the dermis. The tendon in this location did form, but was markedly reduced in size, and no sesamoid appeared to differentiate within it (**Figure 5, (b)**). As development progressed, the tendon degenerated, leaving only disorganized fibrovascular connective tissue in its place (**Figure 5, (d)**).

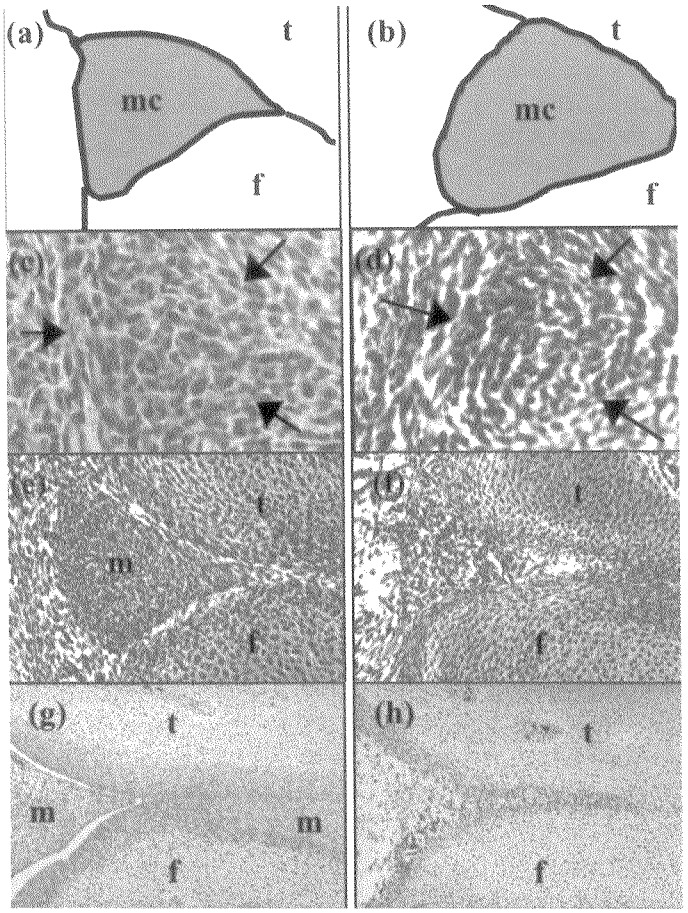

Figure 4.
Meniscus development in control (left) and immobilized (right) chick embryo tibiofemoral joints. Schematic (a) and histologic section (b) of Day 8 meniscal condensation from a control joint (mc=meniscal condensation, t=tibia, f=femur). Arrows point to the margins of the condensation. Day 8 immobilized embryo schematic and histologic section are shown in (b) and (d), respectively. Day 11 meniscus in control (e) and immobilized (f) tibiofemoral joints (m=meniscus). Note degeneration of the immobilized meniscus on the left-hand side of panel (f). Day 13 meniscus from control (g) and immobilized (h) embryos. Note completely absent meniscus in the immobilized joint, in addition to fusion of the tibia and femur across the presumptive joint line. (6-μm paraffin sections stained with fast green, hematoxylin, and safranin O).

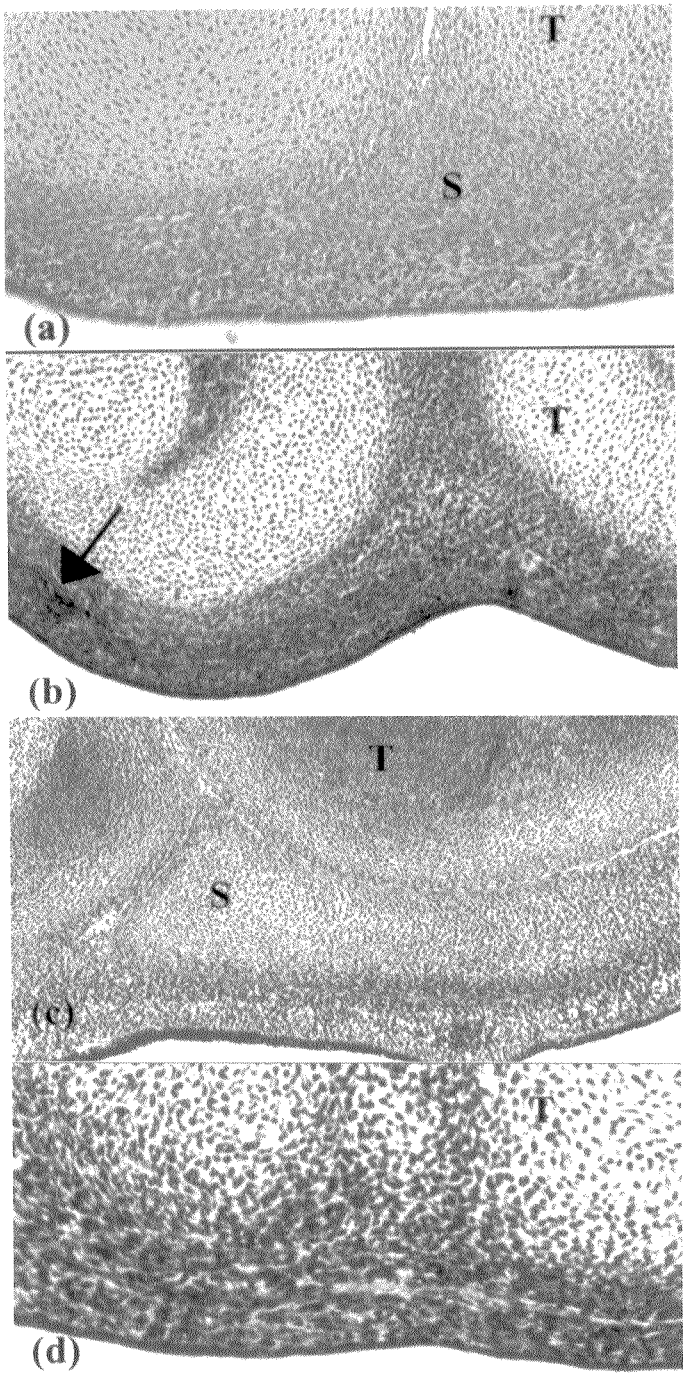

Figure 5.
Sesamoid development in control (a and c) and immobilized (b and d) chick embryo tibiotarsal joints. Day 9 (a and b) and Day 11 (c and d) joints. (S=sesamoid, T=tibiotarsus). Note the absence of an early sesamoid in the tendon of the Day 9 immobilized joint (tendon is demarcated with an arrow at the bottom left of panel) (b) compared to the early-stage sesamoid in the control joint (a). Also note absence of the tendon and continued lack of formation of the plantar tarsal sesamoid by Day 11 (d). (6-μm paraffin sections stained with fast green, hematoxylin, and safranin O).

DISCUSSION

This study demonstrates that embryonic immobilization differentially affects the development of two distinct fibrocartilaginous structures in the chick. Both the menisci and the plantar tarsal sesamoid are completely absent from late-stage immobilized embryos. In contrast to the failure of initial formation of the sesamoid, the early stages of meniscal formation are evident. Without the normal mechanical stimuli of skeletal muscle contractions, however, the meniscus fails to mature and ultimately degenerates. These observations suggest that mechanical loading is integral to the formation of sesamoids. Such structures appear to be the result of tissue differentiation in response to a particular mechanical environment. Meniscal formation, however, bears more resemblance to the other events of synovial joint formation in which the early stages appear to be intrinsically regulated, but the later stages of cavitation and maintenance require functional mechanical stimuli in order to occur.

In tendons that wrap around protrusions of bone, either sesamoid bones or local fibrocartilaginous areas within the tendon are observed (16). Using a single-phase elastic finite element model, Giori and colleagues (15) demonstrated that fibrocartilaginous regions within tendons appear to form in areas of high hydrostatic compression. This phenomenon of chondrometaplasia thus appears to be a mechanically modulated event in which the fibroblasts of the tendon adapt to increased levels of hydrostatic compressive stress by producing extracellular matrix molecules that are more characteristic of cartilage. For example, Vogel and colleagues (17) have demonstrated that cells in the fibrocartilaginous regions of bovine deep flexor tendons express high levels of aggrecan and type II collagen. By increasing the production of such cartilage-specific molecules, the local environment has been adapted in such a way as to ensure that the matrix is better able to support the high compressive stresses resulting from compression against adjacent bone elements. Our study suggests that tissue differentiation in response to local mechanical stresses (much like the chondrometaplasia seen in adults) may be responsible for the initial formation of sesamoid structures in developing avian embryos.

In contrast, the early stages of meniscus formation appear to be intrinsically regulated. Even in the absence of functional skeletal muscle contractions, the early meniscal condensations form. Without normal mechanical stimuli, however, the menisci degenerate and completely disappear. The degenerative response of meniscal tissue to immobilization has been previously demonstrated in postnatal animals (18), but this is the first time that it has been carefully examined during embryogenesis.

The concept of finite windows of opportunity during which environmental factors can modulate developmental fate is worthy of consideration. To truly understand mechanobiological influences on joint development, it would be enlightening to examine the extent to which the effects of immobilization are reversible. Pharmacologic agents other than decamethonium bromide can induce temporary paralysis. Is it possible to reverse the immobilization-induced degenerative changes of the meniscus by remobilizing the embryo? If so, is there a stage after which recovery and regeneration of the meniscus are no longer possible? What molecular events govern this window of opportunity? Such explorations would not only improve our understanding of the role of mechanical factors in joint development, but might also have important implications for optimizing tissue engineering approaches to the repair of joints and joint-associated structures.

As the number of individuals suffering from degenerative joint diseases increases, we are faced with the need for additional approaches to restoring functionality in this patient population. Increasing efforts have focused on the role of biological manipulation of cartilage and other joint-associated tissues. However, the role of mechanical factors (and, more importantly, the interaction of biologic and mechanical factors) must be examined in greater depth. By increasing our understanding and awareness of mechanobiologic influences during development, novel approaches to manipulating the mechanical environment to achieve effective repair of synovial joints and joint-associated structures in adults may someday be possible.

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