Distribution and Progression of Chondrocyte Damage in a Whole-Organ Model of Human Ankle Intra-Articular Fracture

By Yuki Tochigi, MD, PhD, Joseph A. Buckwalter, MS, MD, James A. Martin, PhD, Stephen L. Hillis, PhD, Peng Zhang, MD, Tanawat Vaseenon, MD, Abigail D. Lehman, BS, and Thomas D. Brown, PhD

Investigation performed at The University of Iowa, Iowa City, Iowa

Background: Despite the best current treatments, intra-articular fractures commonly cause posttraumatic osteoarthritis. In this disorder, death and dysfunction of chondrocytes associated with acute cartilage injury presumably plays an important role in triggering the pathomechanical cascade that eventually leads to whole-joint degeneration. Information regarding this cell-level cartilage injury, particularly at the whole-organ level in actual human joints, has been lacking. In this study, the distribution and progression of fracture-associated cell-level cartilage damage were assessed using a novel whole-organ model of human ankle intra-articular fracture.

Methods: Seven normal human ankles harvested immediately following amputation were subjected to a transarticular compressive impaction insult that mimicked an injury mechanism typical of tibial plafond fractures. For each ankle, site-specific, time-dependent changes in chondrocyte viability in the fractured tibial surface were studied by means of live-dead assay, using a confocal laser-scanning microscope. Fractional chondrocyte death was measured at several time points, in the superficial zone of the cartilage in “fracture-edge” regions within 1 mm of the fracture lines, as well as in “non-fracture” regions more than 3 mm centrally away from the fracture lines.

Results: All seven experimental fractures morphologically replicated tibial plafond fractures. Immediately post-fracture, superficial-zone chondrocyte death was significantly greater ($p = 0.001$) in fracture-edge regions (fractional cell death = 7.6%) than in non-fracture regions (1.6%). Progression of cell death over the next forty-eight hours was significantly faster in fracture-edge regions ($p = 0.007$), with the fractional cell death reaching 25.9%, which was again significantly higher ($p < 0.001$) than in non-fracture regions (8.6%).

Conclusions: Cell-level cartilage damage in human intra-articular fractures was characterized by acute chondrocyte death that predominated along fracture lines and that spontaneously progressed in the forty-eight hours following injury.

Clinical Relevance: Progressive chondrocyte damage along fracture lines appears to be a reasonable target of therapeutic treatment to preserve the whole-joint cartilage metabolism in intra-articular fractures, eventually to mitigate the risk of posttraumatic osteoarthritis.

Intra-articular fractures commonly cause posttraumatic osteoarthritis (OA), despite current treatments that effectively reduce and stabilize the fractured surfaces. Clinical series of tibial plafond fractures¹⁻⁴ have shown that more than half of patients treated by surgical reconstruction of the joint nevertheless develop posttraumatic OA, often within two years of injury. To develop novel treatment strategies that effectively mitigate the risk of posttraumatic OA, there is a clear need to

advance the understanding of the pathogenesis of progressive joint degeneration following injury.

There are several factors that presumably contribute to cartilage degeneration in fracture-injured joints^{5,6}. These factors include acute mechanical articular cartilage damage at the instant of the injury event, biological stimuli associated with joint bleeding and/or inflammatory responses, and chronic cartilage overloading resulting from incongruity, instability,

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and malalignment. Recently, chondrocyte damage involved in human intra-articular fractures has been receiving increasing attention⁷⁻⁹. Death or dysfunction of chondrocytes in fracture-damaged joints necessarily impacts cartilage metabolism, presumably triggering a pathological cascade that eventually leads to whole-joint degeneration. Cell-level cartilage injury appears to be an important pathological process to target for new orthopaedic treatment(s) to forestall the development of OA following intra-articular fractures.

Previous studies documented that chondrocyte damage accompanying fracture-associated articular cartilage injury in humans was distributed near the edges of fracture fragments, particularly in the superficial zone^{7,8}. Unfortunately, in these studies, the use of tissue material obtained at the time of definitive fracture surgery precluded exploration of the characteristics of cell-level cartilage damage occurring in the early acute phase, due to probable post-injury damage progression. In addition, chondrocyte viability in non-fracture regions could not be assessed.

The aim of the present study was to document the distribution and progression of chondrocyte damage in fracture-injured human articular surfaces, at the whole-organ level. The joint of interest was the ankle, in which a relatively high prevalence of intra-articular fractures and predictable OA development had been clinically well documented¹⁻⁴. A unique model of human distal tibial intra-articular fracture was developed to study site-specific, time-dependent changes in chondrocyte death in fractured cartilage in the early acute phase. It was hypothesized that acute chondrocyte damage in human intra-articular fractures would be concentrated along fracture lines and that this chondrocyte damage would progress in the forty-eight hours after injury.

Materials and Methods

Seven normal human ankles obtained immediately (<4 hours) following surgical amputation, from patients (four men and three women, twenty-nine to seventy-one years old) who had a malignant tumor near or proximal to the knee, were utilized. In every case, the specimen was harvested from discarded tissue after pathological examination, with the only information collected being the age and sex of the patient. The tibial shaft, sectioned at the mid-diaphysis, was potted perpendicularly into a poly(methyl-methacrylate) (PMMA) block, leaving 10 cm of the distal part intact (Fig. 1). The subtalar joint was disarticulated, and the bottom of the talus was potted into another PMMA block. During this potting procedure, the ankle was held in the neutral position. Special attention was paid to align the center of the ankle to both potting blocks. The proximal fibular shaft (sectioned 9 cm above the ankle) and the lateral malleolar tip were kept away from the potting blocks. The skin and subcutaneous soft tissue surrounding the ankle was left intact.

These ankles were subjected to an impactation insult that mimicked an injury mechanism typical of clinical tibial plafond fractures. Specifically, the ankle was subjected to a high-energy trans-articular compressive force pulse delivered by a custom drop-tower impactation system (Fig. 1). In this device, each specimen was mounted with the tibial side down, and an aluminum impact interface was attached to the upper face of the talar potting block. The specimen was aligned carefully with the axis of mass fall, and secured to the massive base plate, with the talar impact interface held horizontal. A 6.55-kg mass was then dropped from a 78-cm height, delivering approximately 50 J of kinetic energy to the specimen.

Immediately after impactation, each ankle was disarticulated, and the distal tibial articular surface was digitally photographed (Fig. 2) to record the

Fig. 1

A human ankle whole-joint specimen prepared for the fracture-impactation insult (left), and the custom drop-tower system to deliver a fracture-impactation insult (right). An electromagnet holds and releases the drop-mass from a prescribed height.

fracture morphology. Next, under sterile conditions, major osteoarticular fragments were sampled, with juxta-articular bone being trimmed away, while leaving approximately a 5-mm thickness of cancellous bone beneath the cartilage layer (the typical volume per specimen was 1 to 2 cm³). Each osteo-articular fragment was then secured to a specimen holder, such that the articular surface near the fracture edge was held nearly horizontal, by potting in a biocompatible polymer (Polycaprolactone; Sigma-Aldrich, St. Louis, Missouri). The specimens were then immersed in culture medium (approximately 80 mL per specimen), which was comprised of 45% Ham F-12 Nutrient Mixture, 45% high-glucose Dulbecco modified Eagle medium, and 10% fetal bovine serum (all from Invitrogen, Carlsbad, California), with a 1% antibacterial-antifungal mixture of penicillin-streptomycin (Invitrogen) and amphotericin B (Thermo Scientific HyClone, Logan, Utah), in an incubator maintained at 37°C in an atmosphere of 5% CO₂ in air.

Chondrocyte death in these osteoarticular fragments was assessed with use of calcein AM for labeling live cells and ethidium homodimer-2 for labeling dead cells (Invitrogen). Prior to analysis, the fragments were soaked in culture medium with these fluorescence stains (at a concentration of 1.0 μM each) for approximately one hour at 37°C. After labeling, chondrocytes in the superficial zone, from the cartilage surface to a depth of up to 200 μm (typically 100 to 150 μm), were scanned at 20-μm intervals, using a confocal microscopy system (MRC-1024; Bio-Rad Laboratories, Hercules, California), with the specimen kept immersed in the regular medium during scanning (at room temperature, one to two hours per specimen). After each scanning session, the specimens were washed with Hank's balanced salt solution (#14170; Gibco-Invitrogen, Grand Island, New York) containing 1% of the above-described antibacterial-antifungal mixture and placed into fresh culture media.

Fig. 2

Distal tibial intra-articular fractures created in the seven human ankle specimens immediately following surgical amputation.

The first two ankles were scanned for cell viability from six to eight hours after the fracture insult. For these ankles, scans were executed at several sites along the primary fracture lines within 1 mm of the fragment edges (fracture-edge regions) and in the regions at least 3 mm centrally (in a direction perpendicular to the fragment edge) away from the fragment edges (non-fracture regions), with use of a $\times 10$ objective lens that allowed scanning of an approximately 1080×1080 - μm square field per site. The third ankle fracture was scanned after two days of culture. For this ankle, prior to scanning, five representative sites along the primary fracture lines were selected in fracture-edge regions. For each of these sites, two contiguous 540×540 - μm fields (one including a fracture edge and the next centrally adjacent image field) were scanned with use of a $\times 20$ objective lens (Fig. 3). Scans in non-fracture regions were performed at two nonadjacent sites selected near each fracture-edge scan site but at least 3 mm centrally away from the fragment edges. Scanning at any sites with macroscopically identifiable structural damage (e.g., visible superficial cracks) was avoided. The remaining four ankles were scanned at three time

points: immediately (scans completed within six hours), at one day, and at two days post-fracture, in a manner analogous with the third ankle. Prior to the immediate scanning, four to six sets of scanning sites were selected with use of the above-described protocol, and the x-y coordinate information was recorded. Based on this coordinate information, scans at the later time points were repeated at the identical sites, with use of a custom-built programmable microscope x-y axis stage driver (positioning reproducibility, <25 μm).

The scanned images were analyzed with use of ImageJ software (National Institutes of Health, Bethesda, Maryland). Image data for each site and time point consisted of two sets of multi-slice images, one for live cells labeled by green fluorescence and the other for dead cells labeled by red fluorescence. The images, originally in grayscale, were converted to a binary format, allowing fluorescent-labeled cells to be counted with use of the software's particle analysis function. For every image set for live cells, the image intensity threshold for the binary conversion was adjusted such that all plausibly labeled cells were counted, while paying attention to avoid counting the same cells over neighboring multiple

Fig. 3

An osteoarticular fragment mounted in the stage-driver system (left), and an example of scan site selection (right).

TABLE I Individual Specimen Information and Fractional Cell-Death Data

Case (Age, Sex)	Number of Fragments Harvested	Number of Scan Sites	Fracture-Edge Region		
			Mean Fractional Cell Death (Range) (%)		
			Day 0	Day 1	Day 2
#1 (in 40s, M)	2	3	4.9 (0.0 to 14.5)	—	—
#2 (in 50s, M)	2	2	1.2 (0.1 to 2.2)	—	—
#3 (57, F)	5	5	—	—	13.6 (2.8 to 28.9)
#4 (29, F)	4	5	11.5 (4.7 to 29.6)	20.3 (0.8 to 41.8)	25.5 (4.6 to 58.1)
#5 (71, F)	3	5	6.1 (1.3 to 15.8)	9.4 (3.5 to 15.6)	40.7 (30.8 to 62.4)
#6 (57, M)	3	6	12.2 (3.7 to 18.4)	26.7 (10.4 to 47.9)	34.0 (14.0 to 58.7)
#7 (68, M)	2	4	6.2 (3.5 to 10.0)	7.4 (3.1 to 11.5)	14.0 (10.0 to 18.8)

slices. The identical binary conversion threshold was utilized for cell counting for the corresponding dead-cell image set. The minimum particle size thresholds for automated cell counting were 50 and 20 μm^2 for live and dead cells, respectively. For each site and time point, live and dead cells were counted separately, and the fraction of dead cells among total cells was computed.

In statistical analysis, to account for the repeated-measures and unbalanced-design characteristics of the data, a repeated-measures analysis of variance was performed by fitting a general linear mixed-effects model with use of the SAS PROC MIXED procedure (SAS 9.1.3; SAS Institute, Cary, North Carolina). This estimation approach allowed for unbalanced data. Specifically, each specimen could have a different number of observations at each time point and/or could have no observations at one or more time points¹⁰. The MIXED approach also afforded flexibility in the specification of the variance-covariance structure. Variances for each location/day combination were allowed to differ. Covariance within specimens (i.e., for a pair of observations from the same specimen, but from different location/day combinations) was modeled by including a random subject effect, which resulted in the same within-specimen covariance between two location/day combinations. Since the variances were allowed to differ, the within-specimen correlations differed although the within-specimen covariance was constant. (Note: the [adjusted] means and 95% confidence intervals reported in the Results section are based on this model; these adjusted means are not conventional arithmetic means of the subject-level observations, but rather are weighted optimally by the MIXED procedure to take into account correlations, variance differences, and sample-size differences.) All tests were performed with a significance level of alpha set at 0.05. For descriptive purposes, the interquartile ranges (IQRs) in the form (25th percentile, 75th percentile) based on the raw data (i.e., the percentiles are computed as though the data values are independent) were also reported.

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Results

In all seven of these human ankles, comminuted fractures at the distal tibial surface occurred with the single impaction insult, with the fracture morphology (Fig. 2) being consistent with clinical tibial plafond fractures. Prior examination of these joints revealed no appreciable cartilage degeneration or synovitis in any of the specimens.

Fractional chondrocyte death immediately post-fracture impaction was measured at twenty-four fracture-edge sites and at

forty-five non-fracture sites across six ankles. Cell death ranged from 0.0% to 29.6% (IQR: 2.9% to 12.4%) in fracture-edge regions and from 0.0% to 34.2% (IQR: 0.0% to 2.8%) in non-fracture regions, variable across both sites and ankles (Table I). However, the mean value in fracture-edge regions (7.6%, confidence interval [CI]: 4.0% to 11.3%, Fig. 4) was significantly higher ($p = 0.001$) than in non-fracture regions (1.6%, CI: 0.0% to 4.7%). Cell death at two days post-fracture was assessed at twenty-five fracture-edge sites and at forty-seven non-fracture sites, across five ankles. Cell death ranged from 2.8% to 62.4% (IQR: 12.4% to 38.6%) in fracture-edge regions and from 0.0% to 68.8% (IQR: 0.8% to 11.5%) in non-fracture regions. Again, the mean value in fracture-edge regions (25.9%, CI: 18.7% to

Fig. 4
Chondrocyte viability in fracture-edge regions vs. non-fracture regions, immediately and at two days post-fracture. The values of percent fractional cell death are given as (adjusted) means calculated in the MIXED procedure. The dispersion bars indicate 95% confidence intervals.

TABLE I (continued)

Number of Scan Sites	Non-Fracture Region		
	Mean Fractional Cell Death (Range) (%)		
	Day 0	Day 1	Day 2
2	0.1 (0.1 to 0.2)	—	—
2	1.3 (0.0 to 2.6)	—	—
10	—	—	7.5 (0.2 to 32.8)
8	0.8 (0.0 to 5.6)	0.5 (0.0 to 1.2)	4.8 (0.0 to 25.0)
15	0.9 (0.0 to 3.1)	2.4 (0.0 to 11.8)	11.3 (0.0 to 37.6)
12	5.4 (0.2 to 34.2)	13.6 (0.6 to 55.6)	14.9 (1.1 to 68.8)
8	2.0 (0.0 to 4.4)	1.7 (0.0 to 6.2)	1.2 (0.0 to 5.9)

33.1%) was significantly higher ($p < 0.001$) than it was in non-fracture regions (8.6%, CI: 3.4% to 13.8%).

The three-time-point chondrocyte death analysis was performed at twenty fracture-edge sites and at forty-one non-fracture sites across the last four of the tested seven ankles. Cell death in fracture-edge regions, whose mean was 9.1% (CI: 4.0% to 14.2%) immediately post-fracture, increased to 16.6% (CI: 9.4% to 23.8%) at one day post-fracture ($p = 0.040$, vs. immediately post-fracture), and further increased to 29.2% (CI: 20.6% to 37.9%) at two days post-fracture ($p = 0.015$, vs. one day post-fracture) as shown in Figures 5 and 6. Although cell death in non-fracture regions also increased

from immediately to two days post-fracture ($p = 0.014$), the fractions remained relatively low, below 10% throughout the test period in the majority (76%) of the non-fracture scan sites, and neither of the one-day comparisons (one day vs. immediately post-fracture and two days vs. one day) attained significance. The result of repeated-measures two-way analysis of variance revealed that progression of chondrocyte death from immediate to two days post-fracture in fracture-edge regions was significantly faster than in non-fracture regions ($p = 0.007$), with the near-fracture mean change at 20.1% (CI: 11.6% to 28.7%) and the non-fracture change at 6.3% (CI: 1.3% to 11.3%).

Fig. 5

Fig. 6

Fig. 5 Time-dependent changes in human chondrocyte viability at fracture-edge regions and non-fracture regions. The values of percent fractional cell death are given as (adjusted) means calculated in the MIXED procedure. The dispersion bars indicate 95% confidence intervals. **Fig. 6** Confocal microscope images of human superficial chondrocyte viability at a representative fracture-edge scan site, at the immediate, one day, and two days post-fracture time points. Live cells are labeled by green fluorescence, while dead cells are labeled by red fluorescence. White arrows indicate the edge of cartilage on the fracture line.

Discussion

To the best of our knowledge, this is the first study of the distribution and progression of whole-joint chondrocyte death in human intra-articular fractures, in which both specimens and an insult method have closely replicated conditions in human clinical intra-articular fractures. Specifically, fracture-associated articular cartilage pathology was modeled in fresh human cartilage in an anatomical location that clinically frequently sustains intra-articular fracture. For every ankle tested, the fracture insult was delivered within four hours after surgical amputation, and with the joint capsule having been kept closed. Given that articular cartilage is avascular, the consequence of this relatively short duration of blood supply disruption on chondrocyte biological activities was minimal, as confirmed by the immediate low fractional chondrocyte death in the non-fracture regions. It therefore seems reasonable to regard these experimental conditions, under which articular cartilage in these joints was fractured, as approximating those in closed human intra-articular fractures *in vivo*. Nevertheless, a limitation of this study is the post-fracture conditions, including the lack of blood supply and synovial and cellular reaction to the injury. These otherwise may contribute to subsequent progression of chondrocyte death. However, an advantage of the lack of these “extrinsic” biological influences following injury is that it was possible to study the isolated effect of mechanical insult to the articular surface. Another potential limitation is that, since the ankles utilized were from patients who had malignant tumor, these joints may have been load-protected for a certain period prior to amputation. Systemic chemotherapy before surgery also might have influenced chondrocyte activity. However, cells in non-fracture regions retained high fractional viability at most sites even at two days post-fracture, suggesting that the articular cartilage in these joints was initially reasonably healthy. Similarly, the potential adverse effects of *ex vivo* experimentation on chondrocyte viability were also presumed to be noncritical. A strength of this study was that the insult methodology utilized to produce the fractures in these joints mimicked an injury mechanism typical of clinical tibial plafond fractures. Given that the fractures introduced were morphologically consistent with this type of fracture as seen clinically, the physiologic clinical injury mechanism arguably was appropriately replicated in this model.

The appreciable chondrocyte death identified along fracture lines in the present study is consistent with observations in previous studies that explored fracture-associated cartilage pathology in small osteoarticular fragments during surgery for intra-articular fracture fixation⁷⁻⁹. The low fractions of cell death across non-fracture regions, documented in our study, indicate that chondrocyte death in fractured human joints occurs predominantly near fracture lines. Chondrocyte damage in intra-articular fractures appears to be closely associated with structural damage of the articular surface, just as suggested by cartilage blunt-impact experiments^{11,12} and by blunt-wounding experiments^{13,14} (in which blunt wounding introduced more severe chondrocyte damage than a sharp scalpel cut). Assuming that the majority of chondrocyte dam-

age occurs along fracture lines, the degree of whole-joint chondrocyte dysfunction would presumably be correlated with the total length of fracture lines, which in turn correlates with mechanical energy absorption during the fracture event^{15,16}. This observation supports the concept that joints with more severely comminuted articular surfaces sustain a greater insult to cartilage metabolism, and therefore are at a greater risk of posttraumatic OA.

The measured time-dependent increase of cell death in fracture-edge regions indicates that chondrocyte damage in these fractures propagates spontaneously with time. The chondrocyte death identified immediately post-fracture was presumably cell necrosis, due to excessive acute mechanical stresses from the fracture. These physical stresses might have caused delayed apoptosis, which would have contributed to the acute-phase propagation of chondrocyte damage identified at the later observation times. Another possible contributing factor is the cytotoxic effect of biochemical mediators released from damaged chondrocytes and/or from the disrupted extracellular matrix, similar to that observed in cartilage-explant blunt-impact studies¹⁷⁻¹⁹. In intra-articular fractures *in vivo*, acutely damaged cartilage is also subsequently subjected to biological stimuli associated with joint bleeding and/or inflammatory responses, including factors known to accelerate cartilage degeneration, such as reactive oxygen species, matrix-damaging enzymes, inflammatory cells, and inflammatory cytokines²⁰⁻²⁵. Arguably, chondrocyte damage in fractured joints is therefore not fully established at the instant of injury event. There may be a certain window of time during which biological treatment may be able to inhibit progression of chondrocyte damage in the early acute phase, potentially moderating the pathological cascade that leads to posttraumatic OA.

Chondrocyte death that predominated along fracture lines in this study suggests that whole-organ cell-level cartilage damage in a fractured human ankle joint is dependent on the total fracture line length and is therefore closely associated with the initial injury severity. Progression of chondrocyte death during the two days of cartilage culture suggests that the fracture-associated cell-level cartilage damage propagates spontaneously with time during the early acute phase. In the somewhat analogous situations of acute myocardial infarction and ischemic stroke, early thrombolytic therapy to minimize progressive cell death has led to dramatic improvement of clinical outcomes^{26,27}. A similar concept may apply to treatment of intra-articular fracture. Arguably, the most effective treatment to mitigate the risk of posttraumatic OA would not only treat macroscopic damage with anatomical fracture reduction and fixation but would also seek to preserve viable, metabolically effective chondrocytes. ■

Yuki Tochigi, MD, PhD
Tanawat Vaseenon, MD
Thomas D. Brown, PhD

Department of Orthopaedics and Rehabilitation (Y.T., T.V., and T.D.B.)

and Department of Biomedical Engineering (T.D.B.),
The University of Iowa,
2181 Westlawn,
Iowa City, IA 52242.
E-mail address for Y. Tochigi: yuki-tochigi@uiowa.edu

Joseph A. Buckwalter, MS, MD
Department of Orthopaedics and Rehabilitation,
The University of Iowa,
200 Hawkins Drive,
Iowa City, IA 52242

James A. Martin, PhD
Peng Zhang, MD
Abigail D. Lehman, BS
Department of Orthopaedics and Rehabilitation,
The University of Iowa,
1182 ML, Iowa City, IA 52242

Stephen L. Hillis, PhD
The Center for Research in the Implementation of Innovative Strategies
in Practice (CRIISP), VA Iowa City Medical Center, 152 VA,
Iowa City, IA 52242

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