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ORIGINAL ARTICLE

Transglutaminase-2 differently regulates cartilage destruction and osteophyte formation in a surgical model of osteoarthritis

A. Orlandi · F. Oliva · G. Taurisano · E. Candi · A. Di Lascio · G. Melino · L. G. Spagnoli · U. Tarantino

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Abstract Osteoarthritis is a progressive joint disease characterized by cartilage degradation and bone remodeling. Transglutaminases catalyze a calcium-dependent transamidation reaction that produces covalent cross-linking of available substrate glutamine residues and modifies the extracellular matrix. Increased transglutaminases-mediated activity is reported in osteoarthritis, but the relative contribution of transglutaminases-2 (TG2) is uncertain. We describe TG2 expression in human femoral osteoarthritis and in wild-type and homozygous TG2 knockout mice after surgically-induced knee joint instability. Increased TG2 levels were observed in human and wild-type murine osteoarthritic cartilage compared to the respective controls. Histomorphometrical but not X-ray investigation documented in osteoarthritic TG2 knockout mice reduced cartilage destruction and an increased osteophyte formation compared to wild-type mice. These differences were associated with increased TGFβ-1 expression. In addition to confirming its important role in osteoarthritis development, our results demonstrated that TG2 expression differently influences cartilage destruction and bone remodeling, suggesting new targeted TG2-related therapeutic strategies.

Keywords Osteoarthritis · Transglutaminase-2 · Cartilage · Osteophyte · TGF-β1

Abbreviations

FXIIIA	Factor XIIIA
IL-1β	Interleukin-1beta
TGF-β1	Transforming growth factor-β1
TGs	Transglutaminases
TG2	Transglutaminase-2

Introduction

Osteoarthritis is a slowly progressing chronic joint disease that affects most people over the age of 65 (Petersson and Jacobsson 2002). Its etiology is unclear, but it is known to be complex and to involve many cellular and biochemical processes. Given the current trend toward aging of the human population, osteoarthritis, which is age-related (Karlson et al. 2003), will be an increasing economic burden on society. Degradation of articular cartilage and osteophyte formations are major features of osteoarthritis (Petersson and Jacobsson 2002). Synovitis, another important characteristic observed in osteoarthritis, occurs as a secondary inflammatory symptom following cartilage biochemical and mechanical stress (Goldenberg and Cohen 1978). Normal cartilage extracellular matrix is in a state of dynamic equilibrium, with a balance between synthesis and degradation. It is generally believed that, in osteoarthritis, a deregulation occurs of the balance between proteinases that degrade the extracellular matrix and their inhibitors in

A. Orlandi (✉) · A. Di Lascio · L. G. Spagnoli
Anatomic Pathology, Department of Biopathology,
University of Rome "Tor Vergata" School of Medicine,
Via Montpellier, 00133 Rome, Italy
e-mail: orlandi@uniroma2.it

F. Oliva · G. Taurisano · U. Tarantino
Department of Orthopaedics and Traumatology,
University of Rome "Tor Vergata" School of Medicine,
Viale Oxford 81, 00133 Rome, Italy

E. Candi · G. Melino
Experimental Medicine and Biochemical Sciences,
University of Rome "Tor Vergata" School of Medicine,
Via Montpellier, Rome, Italy

favor of proteolysis (Soder et al. 2005). Chondrocytes in normal articular cartilage remain largely in a resting state and do not undergo terminal differentiation (Goldring 2000). In osteoarthritis, foci of chondrocyte hypertrophy develop, typically near sites of cartilage surface damage (Poole et al. 1989). Chondrocyte hypertrophy is likely to promote successive calcification, co-localized with deposits of calcium pyrophosphate crystals (Ishikawa et al. 1989). Transglutaminases (TGs) catalyze a calcium-dependent transamidation reaction that produces covalent cross-linking of available substrate glutamine residues to a primary amino group and modify the extracellular matrix through effects including protein cross-linking and stabilization (Chen and Mehta 1999). TG transamidation catalytic activity has been shown to increase in osteoarthritis in joint cartilages (Rosenthal et al. 1997). Among the TGs family members, in the context of connective tissue formation, TG2 is expressed in cartilage by hypertrophic chondrocytes (Aeschlimann and Thomazy 2000; De Laurenzi and Melino 2001; Nurminskaya et al. 2003). TG2 regulates differentiation, adhesion and migration in several cell types (Fesus and Piacentini 2002). TG2 is an essential mediator of interleukin-1beta (IL-1β)-induced calcification, as well as hypertrophic differentiation and calcification in articular chondrocytes in vivo and in vitro (Aeschlimann et al. 1996; Aeschlimann and Thomazy 2000; Nurminskaya et al. 2003; Johnson and Terkeltaub 2005). However, the role of TG2 in osteoarthritic tissue remodeling in vivo is still uncertain. The transforming growth factor-β1 (TGF-β1) also plays a major role in the remodeling of bone (Marie 1997) and osteophyte formation by stimulating osteoblasts (van Beuningen et al. 1994).

We have focused our attention on TG2 in the attempt to better define the remodeling process in articular tissues in vivo in human osteoarthritis and in mice following surgically-induced knee joint instability in mice. The progression of cartilage destruction and bone remodeling in wild-type and TG2 knockout mice was investigated by histomorphometrical, radiological and immunohistochemical methods. Our results highlight the complex role of TG2 during progressive osteoarthritis.

Materials and methods

Human articular cartilage tissues

Articular cartilage samples (10-mm. diameter and 20- to 40-mm-long) were obtained from the intertrochanteric region of the proximal femur. These were obtained from 10 patients (6 females and 4 males, mean age 73 $\pm$ 1 years). Five patients (3 females and 2 males) underwent hip arthroplasty for severe primary osteoarthritis and five control patients (3 females and 2 males) underwent hip arthroplasty for a pathological fracture of the neck of femur. Hip anteroposterior pelvic radiographs and hip radiographs (with a tube to film distance of 120 cm in all cases) were performed with the feet rotated internally (10° $\pm$ 5°), in order to diagnose the radiological signs of osteoarthritis and the type of fracture before the total hip replacement (Ravaud et al. 1999). All experimental procedures were performed in accordance with local Ethical Committee guidelines.

Generation of transglutaminase-2 knockout mice

Mice deficient for TG2 and wild-type littermates were obtained from G. Melino (University of Rome Tor Vergata, Italy). The mice were from a mixed Svj129/C57Bl/6 background and bred at the local animal facility. PCR analysis confirmed genotype of TG2 knockout and wild-type animals. The mice were fed ad libitum and had free access to drinking water. Male and female mice, equally distributed among the groups, were used for the experiments at the age of 3–4 months. Remaining animals used in this study were purchased from Charles River Breeding Laboratories.

Surgical induction of osteoarthritis

All procedures were performed after local Animal Experiment Committee approval had been granted. TG2 knockout mice (n = 18) and wild-type mice (n = 18) were subjected to a severe osteoarthritis-induced model according to Kamekura et al. (2005). Briefly, the mice were anesthetized with 2-2-2 Tribromoethanol (Avertin, 200 µl/10 g body weight), and their bilateral hind limbs were shaved and prepared for aseptic surgery. Surgical equipment included: a sharpoint 15° 5-mm blade micro-surgical knife, micro-iris scissors, micro-corneal suturing forceps and # 11 and # 15 blades. The right knee joint was exposed and the patellar ligament was transected. Then, the anterior/posterior cruciate ligaments and the medial/lateral collateral ligaments were transected, and the medial/lateral menisci removed using a surgical microscope (Zeiss wild M651). After irrigation with saline to remove tissue debris, the skin was closed with 6-0 vicryl (Ethicon, Edinburgh, UK). During the procedure, close attention was paid not to injure the articular cartilage. The contralateral knee joint was sham-operated through the same approach without any ligament transection or meniscectomy. The mice were euthanized with an overdose of CO_2 at 2, 4 and 8 weeks postoperatively. Successively, complete necropsies were performed and the mice examined for gross abnormalities. For the microscopic examination of the brain, joints, vertebrae, spinal cord, spleen, kidneys, liver, heart, duodenum,

pancreas, lung, thymus, testes, and eyes, 4% formaldehyde fixation was performed followed by paraffin embedding.

Radiological analyses

Radiographs of the murine knee joints were taken in the anteroposterior and lateral projections when the mice were euthanized, using a soft X-ray apparatus with a focus-to-film of 55 cm of distance (General Electric Senograph DMR +; Mo/Mo 14 kvolt/35 sec); the films were manually developed (Kodak Dx 80 and Kodak FX40 fixative) and evaluated by two observers blinded with regard to the experimental group. Inter-observer variability was less than 5%. The objective presence of three typical radiological signs of osteoarthritis, namely subchondral bone sclerosis, osteophytosis and soft tissue calcification, was evaluated by assigning value 1 and 0 to the presence and absence of signs, respectively. The mean value of the sums of these three parameters for each animal was considered.

Histomorphometrical assessment of osteoarthritis

Human articular cartilage samples and murine knee joints were kept in 4% formaldehyde for 24–48 h, and then decalcified in 14% ethylene dinitrilotetraacetic acid in 0.36 M NaOH for 5 days, sampled, dehydrated in ethanol and embedded in paraffin blocks. The joints were embedded in paraffin and 5-μm-thick serial sections were obtained through the entire joint. The slides were stained with Hematoxylin&Eosin and examined at 200× magnification using a Reichert–Jung Polyvar light microscope. Human articular cartilage and osteoarthritis were evaluated according to common international criteria (Collins and McElligott 1960). In murine joints, destruction of cartilage was graded from 0 to 3 as follows: 0, normal appearance; 1, minor destruction of the cartilage surface; 2, focal clear loss of cartilage; 3, extensive loss of cartilage. Osteophyte development was graded as follows: 0, normal appearance; 1, formation of cartilage-like tissues; 2, increase of cartilaginous matrix; 3, ossification. All histomorphometrical evaluations were performed by two observers, under blinded conditions. The inter-observer variability was less than 5%. The scores were calculated as sum of the grades for each section through the whole joint in each mouse and then calculating the average grade for each experimental group.

Immunohistochemical investigation

The slides were heated in a dry oven overnight at 60°C, deparaffinized in xylene and rehydrated in graded concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide and methanol. The slides were rehydrated in phosphate-buffered saline. Non-specific antibody binding was blocked by incubation with normal goat serum (Dako Cytomation, Glostrup, Denmark). Serial sections were incubated with anti-TG2 rabbit polyclonal antibody (dilution 1:75; Covalab, Vinci-Biochem, Florence, Italy) and anti-TGFβ1 goat polyclonal antibody (dilution 1:500, Santa Cruz Biotechnology Inc., CA, USA) for 30 min. The slides were then incubated with biotin-labeled specific secondary antibodies (Dako Cytomation, Glostrup, Denmark), followed by a streptavidin-horseradish peroxidase conjugate. Bound antibody was revealed with the use of the substrate 3,3′-diaminobenzidine. Sections were counterstained with Hematoxylin, washed, dehydrated with graded concentrations of ethanol, cleared in xylene, mounted, and examined at light microscopy. Human breast carcinoma was included with each batch of sections as a positive control. The location of the immunoreactions was determined by comparing each section with the adjacent slice stained with H&E. All procedures were performed at room temperature.

Statistical analysis

The Mann–Whitney U and t Students tests were used for comparison of the mean plus standard error of the mean of the groups. The Wilcoxon matched pair test was used for comparisons at different time-points within a group. P values < 0.05 were considered statistically significant.

Results

Macroscopic and microscopic examination of murine extra-articular tissues

TG2 knockout mice were indistinguishable in size and behavior from wild-type mice and displayed no obvious skeletal abnormalities. There were no abnormalities in total body weight or in the macroscopic and histological appearance of the extra-articular tissues examined in both groups.

Histomorphometrical evaluation of severity of osteoarthritis

Grading for osteoarthritis of human femoral heads was grade IV for the primary osteoarthritis group and grade I–II for patients with a fracture (Collins and McElligott 1960). In mice, surgical induction of joint instability caused the progressive onset of osteoarthritis in both TG2 knockout and wild-type mice (Fig. 1). Lesions were apparent on both the femoral and the tibial segment of the joint. Blinded evaluation of the knee joints that had undergone sham

surgery revealed no osteoarthritis in either group of mice. As reported in Fig. 2, after 2 weeks a slight degree of cartilage destruction was observed in both groups. At 4 and 8 weeks, the degree of cartilage destruction was less in TG2 knockout than that of wild-type mice ($P < 0.05$). On the other hand, osteophyte development was more pronounced in TG2 knockout than that of wild-type mice ($P < 0.05$).

Radiologic evaluation of severity of osteoarthritis in mice after surgical induction of joint instability

All the right knees exhibited anterior subluxation of the tibiae and cartilage destruction and osteophyte formation of the joints. The speed of progression of the radiological signs was time-dependent, as shown in Fig. 3, and there was a significant increase comparing different time-points (wild-type 2 weeks vs. 8 weeks: $P = 0.0004$; TG2 knock-out 2 weeks vs. 8 weeks: $P = 0.0008$). Obviously, significant differences between operated and sham-operated knees were present at all times examined (wild-type vs. sham-operated at 2 weeks: $P < 0.04$, at 4 and 8 weeks: $P < 0.001$; TG2 knock-out vs. sham-operated: $P < 0.04$ at 2 weeks, $P < 0.01$ at 4 and 8 weeks). Subchondral bone sclerosis of the tibial plate was observed at 2 weeks and osteophyte formation started at 4 weeks after surgery in both wild-type and TG2 knockout mice. We did not document any significant statistical differences in the routine radiological evaluation between wild-type and TG2 knockout mice at 2, 4 and 8 weeks after surgery. This was true also comparing single radiologic parameters (not shown).

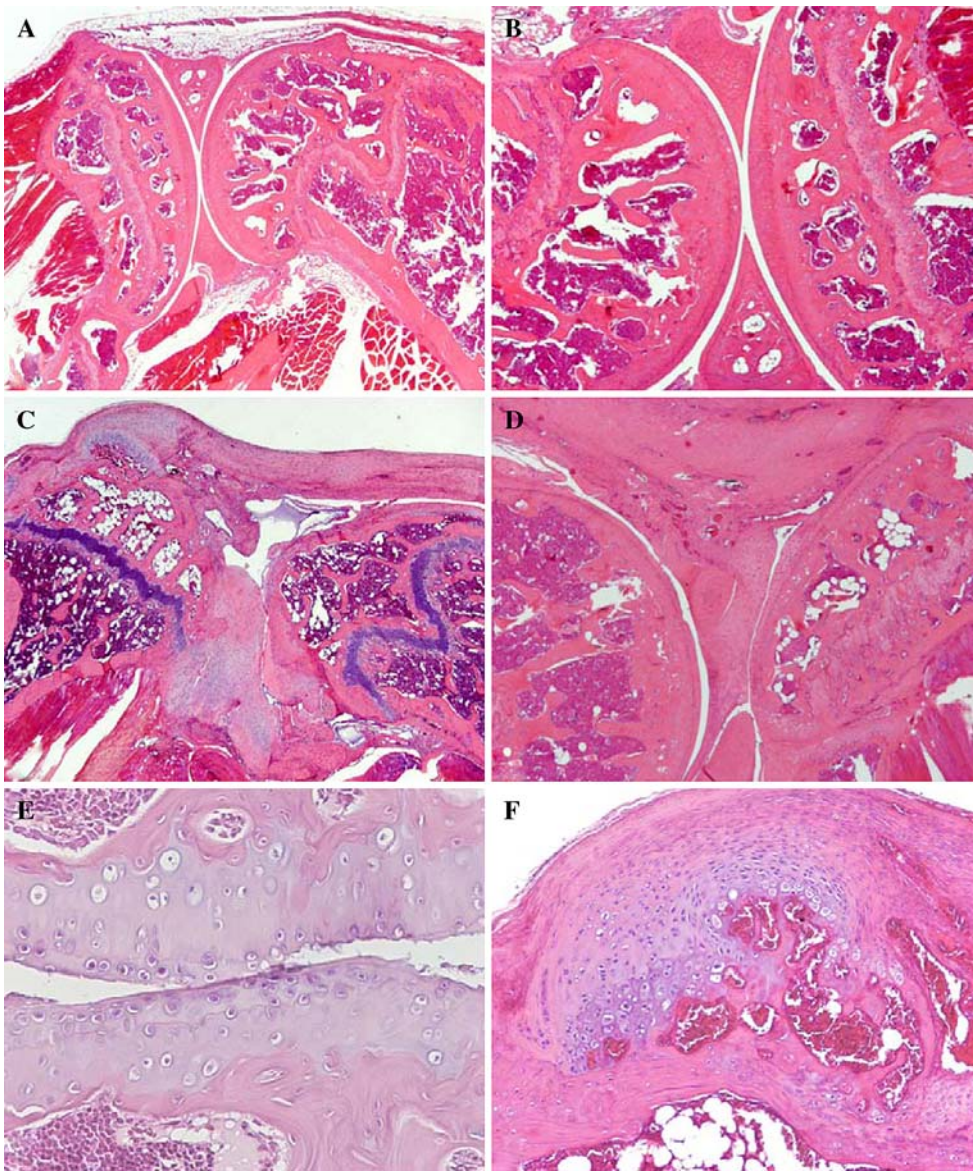

Fig. 1 Knee joint after surgical induction of osteoarthritis in transglutaminase-2 mice. **a**, **b** H&E stained sections of knee joint tissues of sham-operated transglutaminase-2 knockout mice after 8 weeks shows smooth cartilaginous surface of femoral and tibial plates with minimal articular space. **c**, **d** Transglutaminase-2 knockout mice 8 weeks after surgical induction of osteoarthritis shows widening of knee joint articular space with inflammatory tissue, cartilage destruction of both femoral and tibial plates and osteophyte development; **e** at 4 weeks, transglutaminase-2 knockout mice shows minor destruction of articular cartilage; **f** at 8 weeks, osteophyte development involving intra- and periarticular tissues is observed in transglutaminase-2 knockout mice. Original magnification; **a**, **c** ×40; **b**, **d** ×100; **e**, **f** ×200

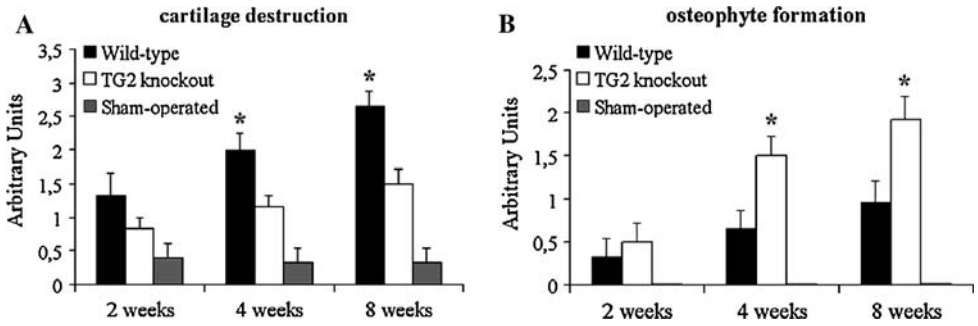

Fig. 2 Histomorphometrical evaluation of knee joint osteoarthritis after surgical induction of osteoarthritis in transglutaminase-2 mice and wild-type mice. Blinded evaluation **a** cartilage destruction and **b** osteophyte formation was performed independently from two researchers and expressed in arbitrary units; results are given as means ± SEM; * $P < 0.05$

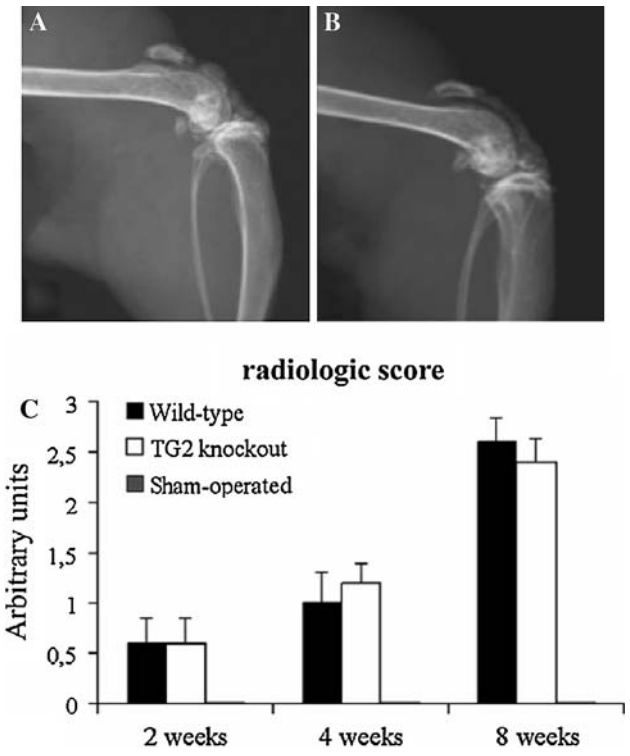

Fig. 3 Evaluation of X-ray features of knee joint osteoarthritis after surgical induction of osteoarthritis in transglutaminase-2 knockout mice and wild-type mice. **a**, **b** X-ray of knee joint of **a** wild-type mice and **b** transglutaminase-2 knockout mice 8 weeks after surgical induction of osteoarthritis. **c** *Bar graphs* showing results of blinded evaluation of X-ray lateral projections for the presence of subchondral bone sclerosis, osteophytes and tissue calcification expressed as sum of arbitrary units. A progressive increased radiological score documents the increase of severity of radiological signs but no significant differences between transglutaminase-2 knockout mice and wild-type mice at examined times

Histomorphometrical evaluation of severity of osteoarthritis

Grading for osteoarthritis of human femoral heads was grade IV for the primary osteoarthritis group and grade I–II for patients with a fracture (Collins and McElligott 1960; Fig. 4). In mice, histomorphometrical evaluation confirmed that surgical induction of joint instability caused the progressive onset of osteoarthritis in both TG2 knockout mice and wild-type mice (Fig. 2). Lesions were apparent on both the femoral and the tibial segment of the joint. Blinded evaluation of the knee joints that had undergone sham surgery revealed no osteoarthritis in either group of mice. After 2 weeks we observed limited cartilage destruction in both groups. At 4 and 8 weeks, the degree of cartilage destruction was less in TG2 knockout mice than that of wild-type mice ($P < 0.05$). On the other hand, osteophyte development was more pronounced in TG2 knockout than that of wild-type mice ($P < 0.05$).

Expression of transglutaminase-2 and TGF-β1

As reported in Fig. 4, TG2 expression was increased in human altered cartilage and other tissues as well as in murine surgically-induced osteoarthritic tissues compared to normal cartilage. Immunohistochemistry confirmed the expression of TG2 in the hypertrophic chondrocytes of wild-type mice, and its absence in those of knockout mice (Fig. 5 a, b). The immunohistochemical investigation also showed an increased TGFβ-1 expression in osteoarthritic tissues compared to sham-operated joints and in human osteoarthritic cartilage compared to controls, according to previous reports (Blaney Davidson et al. 2007). TGF-β1 expression was greater in superficial chondrocytes and in subchondral bone in both groups. TGFβ-1 expression in osteoarthritic and surrounding tissues was greater in TG2 knockout than that of wild-type mice (Fig. 5 B,C).

Discussion

In this paper, we provide new details by means of histomorphometrical methods concerning the contribution of TG2 to the osteoarthritic process. In the latter, chondrocytes

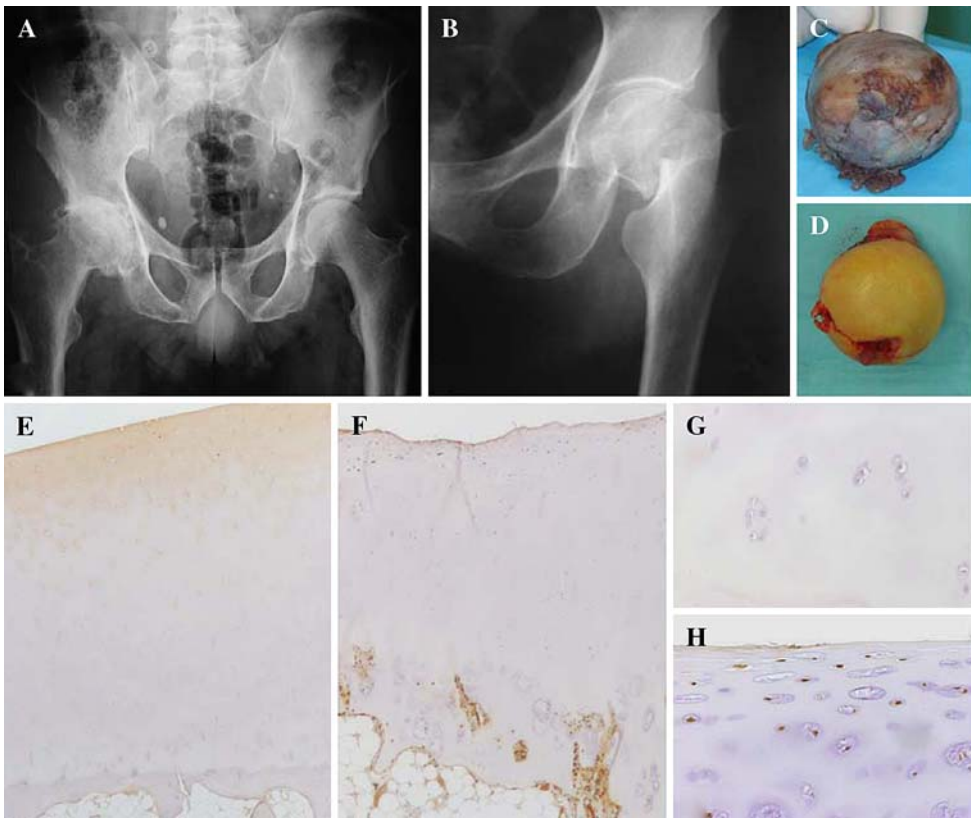

Fig. 4 Osteoarthritis and transglutaminase-2 expression in human proximal femur. **a**, **b** X-ray and **c**, **d** macroscopic features of human proximal femur with **a** radiologic and **c** macroscopic evidence of osteoarthritic degeneration; instead, pathological fracture shows **b** minimal radiologic signs of osteoarthritis and **d** a normal macroscopic appearance. **e–h** Immunohistochemical investigation of femur cartilage at different magnification shows **e**, **g** almost absent chondrocyte transglutaminase-2 expression in femoral cartilage after pathological fracture and **f**, **h** increased transglutaminase-2 expression in the presence of severe osteoarthritis; diaminobenzidine as chromogen; original magnification; **e**, **f** = ×40, **g**, **h** = ×100

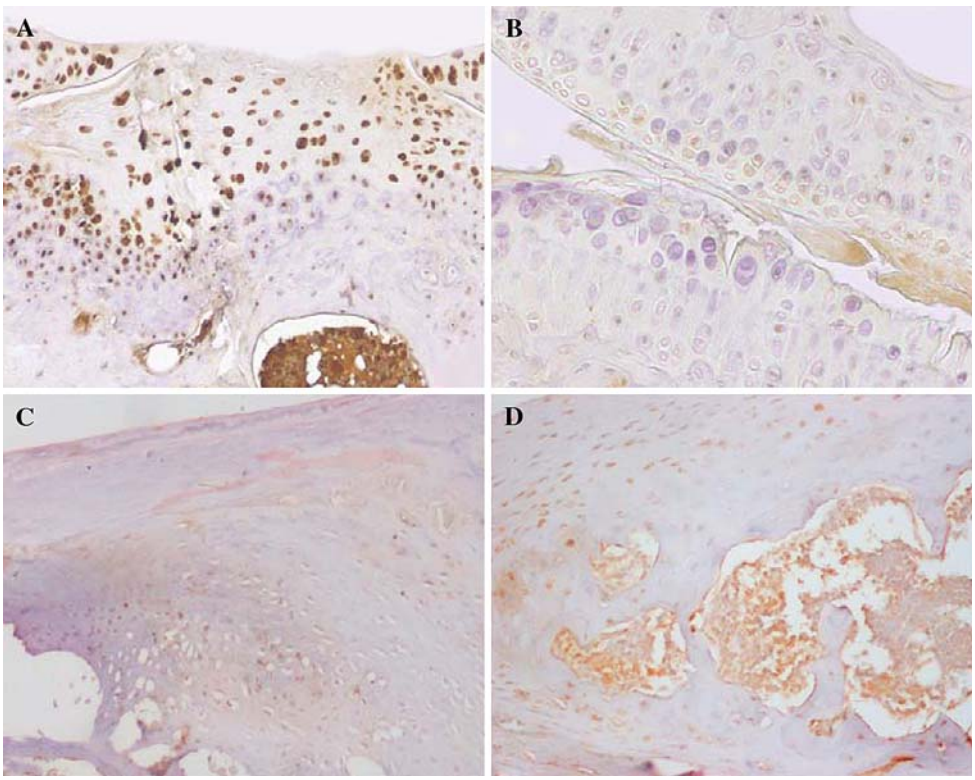

Fig. 5 Transglutaminase-2 and transforming growth factor-β1 expression in murine knee joints after surgical induction of osteoarthritis. Immunohistochemical investigation shows **a** increased chondrocyte transglutaminase-2 expression in the presence of osteoarthritic cartilage destruction in wild-type mice and **b** absent chondrocyte transglutaminase-2 expression in transglutaminase-2 knockout mice 4 weeks after surgical induction of osteoarthritis. At the same experimental time; **c**, **d** immunohistochemical investigation for transforming growth factor-β1 shows less positive immunoreaction in osteoarthritic tissue of **c** wild-type mice compared to **d** transglutaminase-2 knockout mice; diaminobenzidine as chromogen; original magnification; **a**, **b** = ×200; **c**, **d** = ×100

undergo hypertrophy and an accelerated turn-over and, successively, calcification of the pericellular matrix becomes prevalent in sclerotic subchondral bone (Ryan and McCarty 1997). TGs promote normal extracellular matrix mineralization in growth plate cartilage and activate the crystal-promoting factor TGF-β1 (Rosenthal et al. 2000). In particular, TG2 was considered to be a significant factor in promoting calcification in injured chondrocytes in osteoarthritic cartilage, which can contain known inducers of chondrocyte TG activity such as IL-1β, TNF-α and nitric oxide (Johnson et al. 2001).

Our immunohistochemical data confirm that TG2 expression is markedly increased in human and wild-type mice osteoarthritic tissues (Rosenthal et al. 1997). It has been previously reported that TG2, similar to other TGs, catalyzes a post-translational modification of proteins and is associated with biomineralization in growth plate cartilage (Rosenthal et al. 1997). Nevertheless, we documented that TG2 knockout mice also develop osteoarthritic lesions. Since TG2 knockout mice show a normal musculoskeletal apparatus (De Laurenzi and Melino 2001), it is likely that TG2-independent pathways sustain osteocartilaginous growth during bone development and growth as well as the osteoarthritic process. Stimulation in vitro of TG2 knockout mice chondrocytes results in an induction of other TGs, such as factor XIIIA (FXIIIA), but a lack of induction of matrix calcification (Johnson et al. 2003). The existence of redundant TG2-dependent and TG2-independent mechanisms for chondrocyte hypertrophy also explains why normal TG2 knockout mice demonstrated no gross phenotypic abnormalities in their developmental growth plates (Tarantino et al., submitted for publication). As a matter of fact, thrombin treatment of chondrocyte cultures increased FXIIIA mRNA and protein levels, without affecting levels of TG2 (Rosenthal et al. 2004). These data are in line with a distinct role of TG2 in different phases of the osteoarthritic process, in particular the maintenance of cartilage integrity and/or subchondral sclerosis and the development of extra-plate osteophytes. It has been previously reported that TG catalytic activity is diminished by 50% in unstimulated TG2 knock-out mice (Johnson et al. 2003). IL-1b is a contributor to the pathogenesis of osteoarthritis (Attur et al. 1998). Since TG2 catalytic activity or calcification in response to IL-1β in TG2 knock-out mice knee chondrocytes appears reduced (Johnson et al. 2003), it is likely that the underlying terminal chondrocyte differentiation and cartilage matrix calcification process is impaired, keeping chondrocytes in a hypertrophied state and favoring cartilage integrity. As concerns osteoarthritic bone remodeling, it has been recently reported that human osteoblasts from sclerotic areas of subchondral bone show increased TG2 gene expression and reduced matrix mineralization compared to nonsclerotic osteoblasts in vitro (Sanchez et al. 2008). In parallel, protein synthesis of TGF-β1 is significantly higher in sclerotic than in nonsclerotic osteoblasts, while IL-1β production is similar in both groups (Sanchez et al. 2008). Only inhibition of activity of two TG main isoforms, i.e., TG2 and FXIIIA, in pre-osteoblast cultures results in complete abrogation of mineralization and an arrested state of osteoblast differentiation (Al-Jallad et al. 2006). Altogether, these and our present results are in line with the hypothesis of a different role of TG2 in hypertrophied chondrocytes and osteoblasts during the osteoarthritic remodeling process.

In experimentally induced osteoarthritis, reduced cartilage destruction in TG2 knockout mice compared to wild-type mice was associated with the increase of TGF-β1 expression. We also documented a widespread increased TGF-β1 expression in osteoarthritic tissues in TG2 knockout mice compared to wild-type mice. Since endogenous TGF-β1 represents a crucial factor in the process of osteophyte formation, it appears evident that an over-expression of TGF-β1 has an important function during osteophyte development. It has also been reported that blocking of TGF-β receptor II prevents the osteophyte formation process (Scharstuhl et al. 2002). TGF-ß1 is expressed in chondrocytes, osteoblasts and osteocytes and affects many aspects of bone formation (Janssens et al. 2005). In addition, TGF-β1 knockout mice show skeletal defects (Geiser et al. 1998). TGF-β1 influences TGs-catalyzed linkage to the matrix (Nunes et al. 1997). Other studies suggest that the mechanism of TG2-induced mineralization does not involve TGF-β1 (Nurminskaya et al. 2003). Over-expression of TGF-β1 is also likely to maintain chondrocyte hypertrophy and sustain cartilage repair. Moreover, lack of TGF-beta results in osteoarthritis-like cartilage degeneration (Blaney Davidson et al. 2007). It is worth noting that the effects of TGF-β are not limited to cartilage. TGF-β is implicated in fibrosis in many organs (Branton and Kopp 1999). It has been previously reported that TGF-β1 is also able to induce osteophytes similar to those found in osteoarthritis and TGF-β is found highly expressed in osteophytes (Janssens et al. 2005; Blaney Davidson et al. 2007). Our results in osteoarthritic TG2 knockout mice suggest that TGF-β over-expression in osteoarticular and non-cartilaginous tissues helps cartilage integrity maintenance by diffusion but is also responsible for increased fibrosis and extra-plate osteophyte formation.

We did not document any significant statistical differences in the radiological evaluation of osteoarthritic changes between wild-type and TG2 mice at all times examined after surgery, in line with previous reports (Pataki et al. 1990). The absence of correlation between histomorphometrical and routine radiologic investigation depends on the lack of accuracy of the latter in evaluating osteoarthritic changes. New methodologies such as high-resolution

single-photon-emission computed tomography appear to be promising and potentially useful as a diagnostic instrument for monitoring experimental osteoarthritis (Ostendorf et al. 2006).

In conclusion, our results confirm the important role of TG2 in osteoarthritis development and show that endogenous TG2 plays a complex role during osteoarthritic joint tissue remodeling, suggesting new targeted TG2-related strategies for therapeutic interventions.

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