Ex-vivo Evaluation of ^{99m}Tc Labeled Glucosamine Sulfate (^{99m}TcGS) in Articular Cartilage. Relevance for Osteoarthritis Imaging

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Keywords: Cartilage; Chondroitin sulfate; Osteoarthritis imaging; Autoradiography

Abstract

Background: Glucosamine sulfate (GS) is widely used in the treatment of osteoarthritis, but no data concerning the local uptake of GS in articular cartilage exist. For this reason we performed radiolabeling of GS (Rottapharm, Monza, Italy) using the ^{99m}TcO$_4^-$/ tin method and investigated its uptake in articular cartilage.

Methods: Radiolabeling of GS is difficult, because of the small MW of GS (450 Da) and of ^{99m}TcO$_4^-$ for separation after radiolabeling. For uptake studies, articular surgery tissue from patients (n=6, 68-79a) undergoing knee arthroplasty (pieces of ~3 mg wet weight), or frozen tissue sections (10 µ) for autoradiography (10 µCi) were used. The uptake of ^{99m}TcGS was monitored from 0.5 to 72 h of incubation to achieve saturation, by quantitation of radioactivity with a Gamma-counter or autoradiography. To mimic in-vivo treatment, tissue pieces were preincubated with cold GS (80 µg/ml) for 16 weeks prior to incubation with ^{99m}TcGS. The incubation was performed with tissues with different degree of degeneration.

Results: The best radiolabeling results were obtained using 25 mg of GS, 120-150 MBq of ^{99m}Tc, separation on G10 column, specific activity of 162.2-202.8 mCi/mM, n=6. The uptake was time-dependent and amounted to a maximum of 77.4. -71.7% at 48 h versus 72 h, at saturation. Saturation was achieved already after 24 h of incubation. When the tissue was preincubated with cold GS the subsequent uptake of ^{99m}TcGS was strongly reduced by 52.3-71.8%, proving the chondrotropic effects of GS during preincubation. The reduction of ^{99m}TcGS uptake was observed also in areas of different grades of degeneration from the same patient by 27.8-35.8%, proving the specificity of uptake. Autoradiographic studies paralleled the uptake in tissue pieces. We performed a statistical evaluation of differences between all investigated OA patients with different degree of cartilage degeneration. For these patients we evaluated the autoradiographic uptake at all experimental points of incubation times (1 h, 3 h, 6 h, 24 h, 48 h). ^{99m}TcGS uptake in tissues of different extent of degeneration varied significantly during the whole time of incubation, the more degenerated regions showing a significantly higher ^{99m}TcGS uptake. The non-specific uptake in the presence of 50-fold excess of cold GS was increasing with time up to a maximum of about 10%, at saturation.

Conclusions: ^{99m}TcGS could be a suitable tracer to quantify degeneration in articular cartilage, because of its high uptake and specificity. It could be a potential candidate for imaging osteoarthritis in cartilage and possibly for monitoring therapeutic effects.

Introduction

Osteoarthritis (OA) is a degenerative joint disease resulting frequently in a degeneration and irreversible destruction of articular cartilage. Besides collagen (mainly type II), proteoglycans, especially aggrecan, belong to the most abundant components of cartilage. The disease progresses through several phases. In its early stage mainly cartilage proteoglycans (PG) are degraded. As a consequence the quality and quantity of glycosaminoglycan (GAG) side chains are altered. Especially in the superficial zone of OA cartilage, loss of fixed negative charge density (FCD) is manifested. The stationary phase of OA is characterized by the formation of osteophytes and joint space narrowing. The disease finally culminates in the obliteration of the joint space. In general, OA triggers accelerated but inadequate tissue repair. Osteoarthritis involves changes in both synthesis and degradation of aggrecan and other cartilage molecules [1]. PG-degrading enzymes and inflammatory mediators such as cytokines inducing catabolic processes are produced within the cartilage. These mediators and matrix degrading enzymes are generated by cartilage cells, i.e. chondrocytes which may even undergo irreversible phenotypic modulation during the progression of the disease.

Changes in PG content and structure as a consequence of defective biosynthesis and turnover of glucosamine (GA) and other hexosamines occur early in OA. It is therefore tempting to investigate in vivo cartilage uptake of labeled GA as a possible marker for early pathological changes in OA, e.g. by scintigraphic imaging of cartilage. Tissue distribution of uniformly labeled ^{14}C-GA after intravenous or oral administration was studied by Setnikar et al. [2,3]. Ten minutes after i.v. dosage, GA was already found in all organs and tissues, including cartilage. Interestingly, radioactivity in cartilage (femur) was significantly higher than in bone (sternum), suggesting a chondrotropic behavior for GA.

For studies investigating absorption, tissue distribution, excretion and incorporation of GA using scintillation counting, ^{14}C-GA [2,3] or ^{3}H-GA [4] are acceptable. For imaging techniques, however, to study in vivo cartilage degradation, scintigraphy using ^{99m}Tc-labeled

glucosamine sulfate (^{99m}TcGS) as tracer is the method of choice. Labeling with ^{99m}Tc results in the formation of a ^{99m}TcGS-complex, that could be more resistant to metabolism and/or integration into plasma proteins than uniformly labeled ^{14}C-GA or ^{3}H-GA. As the molecular structure of GS is not directly labeled in these complexes, excreted CO_2 or plasma proteins incorporating GS fragments are "silent", both in conventional counting techniques and in scintigraphy. Cartilage associated radioactivity signals are therefore always originating in complex-bound GS.

The present study aims at proving the chondrotropic character of ^{99m}TcGS. We hypothesize that this tracer can be used in the diagnosis of early and advanced OA.

Materials and Methods

Radiolabeling procedure

Radiolabeling of GS (Rottapharm, Monza, Italy) was done using our modified ^{99m}TcO$_4^-$/stannous chloride method [5] at pH 7.0 in 0.01 M citrate for 1 h at room temperature. Separation of labeled GS from unbound ^{99m}Tc was performed by gel chromatography over G 10.

Quality control

For quality control thin layer chromatography (ITLC) was used with the following solvent mixtures: 0.2 M saline/10% ethanol to separate colloid and ethanol/aluminium oxide IB-F to detect free ^{99m}Tc. The latter method was also used to evaluate the stability of the labeled ^{99m}TcGS complex 3 h and 6 h after labeling at room temperature.

Molecular weight estimation of ^{99m}TcGS complex

The molecular weight of the ^{99m}TcGS complex was determined via molecular sieve chromatography using G10 columns (Sephadex, Bio-Rad, California, USA). As molecular weight standards glucose, sucrose and dextran blue were used. The elution position of these sugars and of ^{99m}TcGS complex was detected in the eluted 1 ml fractions on the same gel column. Sugars were quantitated by a colorimetric method [6]. GS measurement in eluted fractions was performed according to the modified method by Elson and Morgan [7]. Molecular weight of the ^{99m}TcGS complex was calculated [8].

Uptake studies

To study the uptake of labeled GS (^{99m}TcGS) by cartilage, articular surgery tissue samples from individuals undergoing knee arthroplasty (n=6, 68-79a) were used. The cartilage sections from different patients were selected according to degeneration or regional variation of degeneration from the same patient. Tissues with different degree of degeneration were evaluated histologically by hematoxylin & eosin staining and uptake was compared. The uptake of ^{99m}TcGS in pieces of ~3 mg wet weight by incubation at 37 °C from 0.5 h to 72 h was monitored by quantitation of radioactivity in the Gamma-counter. After 72 h saturation was achieved. Alternatively, 10 μ frozen tissue sections were incubated with 10 μCi ^{99m}TcGS at 37 °C and uptake was evaluated by autoradiography (Instant Imager, Packard, Meriden, CT, USA). To mimic in vivo therapy with GS treatment, tissue pieces were preincubated with cold GS (80 μg/ml) for 16 weeks (at 37 °C in CGM Bullet medium) prior to incubation with ^{99m}TcGS. This corresponds to a daily dose of 400 mg GS (i.e. one tablet of the drug).

We performed inhibition experiments with cold GS both for higher (grade III) and mildly degenerated samples (grade II). The substrate was renewed every week. To determine the specificity of the binding of the ^{99m}TcGS complex, the degree of uptake inhibition by a 50 fold excess of unlabeled free GS was verified.

Statistical analysis

The uptake data were analyzed for statistical significance using the t-test. Each data point on the plots (Figure 1) represents the mean of the values obtained from 6 independent experiments (6 patients grade II and 6 patients grade III) for each incubation time. Error bars indicating the standard error of means (±SEM) and $p < 0.05$ was considered statistically significant.

Results

Quality control

The radiochemical purity of ^{99m}TcGS amounted to 95% (5% colloid + ^{99m}Tc free, as impurities). Using ITLC-SG-chromatography the radiolabeled complex remained at the start (Rf = 0.2) and free pertechnetate moved with the solvent front (Rf = 0.95). Using aluminium oxide IB-F TLC colloid remained at the start (Rf = 0.1) and the radiolabeled complex moved with the solvent front (Rf = 0.9). The tracers were stable over 6 h after labeling (95% of the radiochemical purity).

Structure of ^{99m}Tc GS-complex

Different labeling procedures were compared. The best results were obtained using a concentration of 25 mg per ml GS, 120-150 MBq of ^{99m}TcO$_4^-$ at a pH of 7.0 in 0.01 M citrate. The ^{99m}TcGS-complex was separated by gel chromatography on Sephadex gel G 10 using PBS as elution buffer. 70.4 % of total radioactivity was bound by the complex. GS was chemically detected exclusively in the main elution peak. The specific activity obtained was 6 MBq per mg ^{99m}TcGS-complex. A molecular weight of approximately 1000 for the labeled complex was computed from the relative elution position of ^{99m}TcGS (Figure 2).

Uptake of ^{99m}TcGS in articular cartilage

To estimate the degree of cartilage degeneration for ex-vivo uptake studies from patients undergoing knee arthroplasty we performed histopathological evaluation of tissue (Figure 3). We selected patients with mild degeneration (grade II) and heavier degeneration (grade III). The use of these samples from patients undergoing knee replacement was approved by the local ethic commission EK-Nr. 523/2008. The uptake in tissue pieces was found to be time-dependent, amounting to a maximum of about 67-78% at saturation, which was already obtained after 24 h of incubation at 37 °C (Figure 4). The reduction of ^{99m}TcGS uptake was observed also in areas of different grade of degeneration from the same patient (Figure 4). ^{99m}TcGS uptake in low degenerated tissue areas (grade II) was lower than in heavier degenerated tissue areas (grade III). Especially in the initial period of incubation up to 18 h we observed a significantly higher uptake of ^{99m}TcGS up to 35.8% in heavier degenerated tissue areas, proving the specificity of uptake for advanced pathological changes (Figure 4).

When degenerated tissues were preincubated with cold GS (80 μg/ml) for 16 weeks to mimic in vivo treatment, followed by
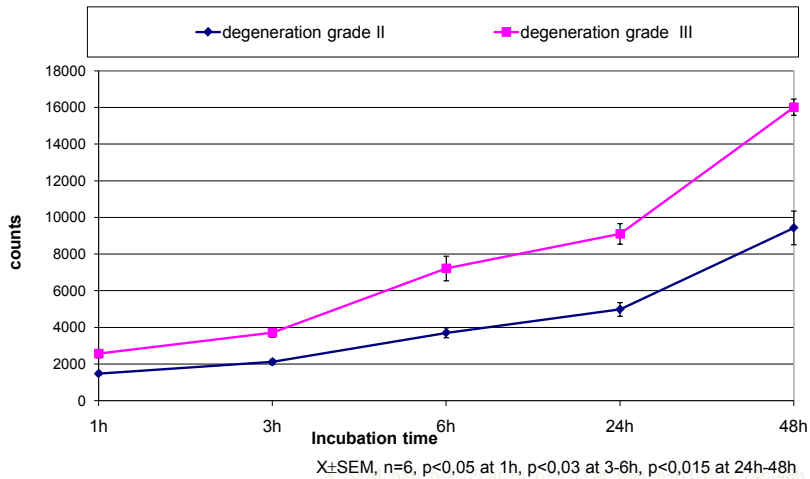
Figure 1: Autoradiographic uptake (total counts) in degenerated human articular cartilage from patients (n=6) with lower degeneration (grade II) versus patients (n=6) with heavier degeneration (grade III). Uptake intensity correlates with degree of degeneration during the whole time of incubation, the more degenerated regions showing a significantly higher 99mTcGS uptake.
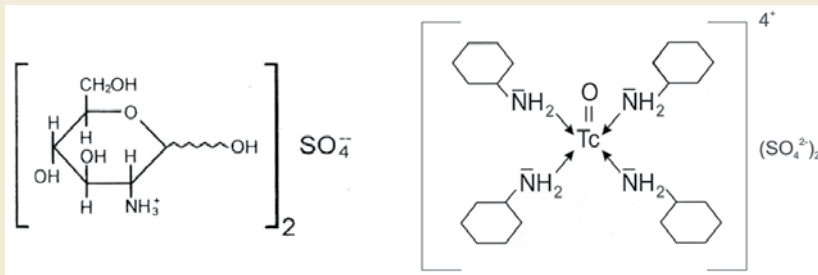

Figure 2: The chemical structure of the starting reagent (glucosamine sulfate) is given left. The proposed structure of 99mTcGS complex formed during the labeling procedure, right. The central 99mTc^{4+} ion is surrounded by four GA molecules forming electron pair bridges via their amino groups. The four ring structure containing amino groups represents symbolically the chemical structure of glucosamine residues (left). The molecular weight of the labeled 99mTcGS complex was verified by molecular sieve chromatography and corresponds to the suggested structure (MW:1000).
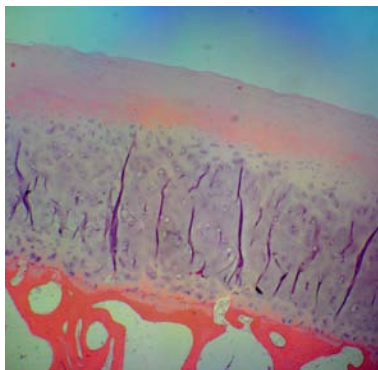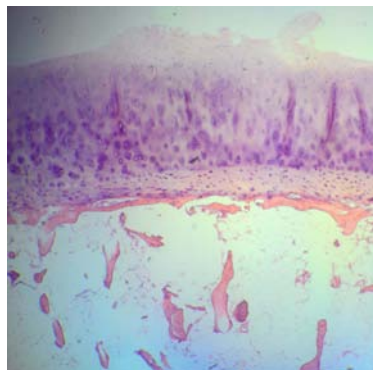
Figure 3: Histopathological examination of degenerated human cartilage tissueby hematoxylin & eosin staining. We selected patient with mild degeneration (grade II) and more severe degeneration (grade III), showing severe erosion of the cartilage surface. Histological pictures of these sections represent magnification of 100x.

incubation with [99m]TcGS the additional uptake of [99m]TcGS (24 h - 72 h) was strongly reduced by 52.3-71.8% (Figure 5a) in degenerated cartilage tissue (grade III). Comparable effect was found in degenerated cartilage tissue (grade II) by 40.3 – 64.3% (Figure 5b). This proves the chondrotropic effects of GS during the treatment. During the incubation with cold GS degenerated cartilage seems to take up GS from incubation medium to repair the cartilage matrix. It is known that in degenerated matrix GAG are depleted and cartilage claims to rebuild GAG in the matrix. This repair mechanism could be observed already after preincubation with cold GS, therefore the additional uptake with [99m]TcGS resulted in lower uptake as compared to incubation with labeled [99m]TcGS alone, without treatment.

Autoradiography

Autoradiographic studies paralleled the uptake by tissue pieces. An example of autoradiographic uptake of [99m]TcGS in patients with different cartilage degeneration is shown in Figures 6 and 7. We performed a statistical evaluation of differences between all investigated OA patients with different degree of cartilage degeneration. For these patients (n=6 for grade II and n=6 for grade III) we evaluated the autoradiographic uptake (counts in cartilage areas) at all experimental points of incubation times (1 h, 3 h, 6 h, 24 h, 48 h) and computed statistical data shown in Figure 1. [99m]TcGS uptake in tissue of different extent of degeneration from patients varied significantly. Uptake intensity correlates with degree of degeneration during the whole time of incubation, the more degenerated regions showing a significantly higher [99m]TcGS uptake (Figure 1). The non-specific uptake in the presence of a 50 fold excess of cold GS was increasing with time up to a maximum of about 10 % at saturation (Figure 8).

Discussion

In the treatment of OA the early recognition of the disease as well as early therapeutic intervention are essential. Among the plethora of drugs used to treat OA, GA belongs to the group of nutraceuticals. Originally only considered to constitute an exogenous source for building blocks in the biosynthesis of PGs, GA, together with chondroitin sulfate (CS) was introduced as safer alternative to anti-inflammatory drugs [9]. The rationale for its use in OA therapy lies in the fact that it is a precursor of GAGs which constitute the side chains of PG representing major components of cartilage. It can reduce inflammation and the extent of cartilage degradation [10,11]. More recently it was found that the combination of GA with CS can enhance these effects [12]. GA is bioavailable after oral administration both systemically and at the site of supposed action, i.e. the joint, persists in circulation and its pharmacokinetics support once-daily dosage [13,14]. GA is known to effectively retard progression of OA, especially on long-term treatment. The drug was also shown to display structure-modifying effects such as reducing joint space narrowing [15-23]. In particular, two recent randomised trials of 2-3 years duration in knee OA patients indicate that these symptom-modifying effects are sustained over long-term treatment [21,23]. Thus, joint space narrowing/radiological progression of OA was found to be significantly reduced on daily GA administration. Hovever, the therapeutical efficacy of GA is controversial [24-26].

Normally, GA and its acetylated bioactive derivative N-acetyl-GA is produced endogenously in sufficient amounts via biosynthesis from glucose. In the presence of exogenous GA, however, biosynthesis of GAGs is enhanced [27]. In general, exogenous GA is incorporated
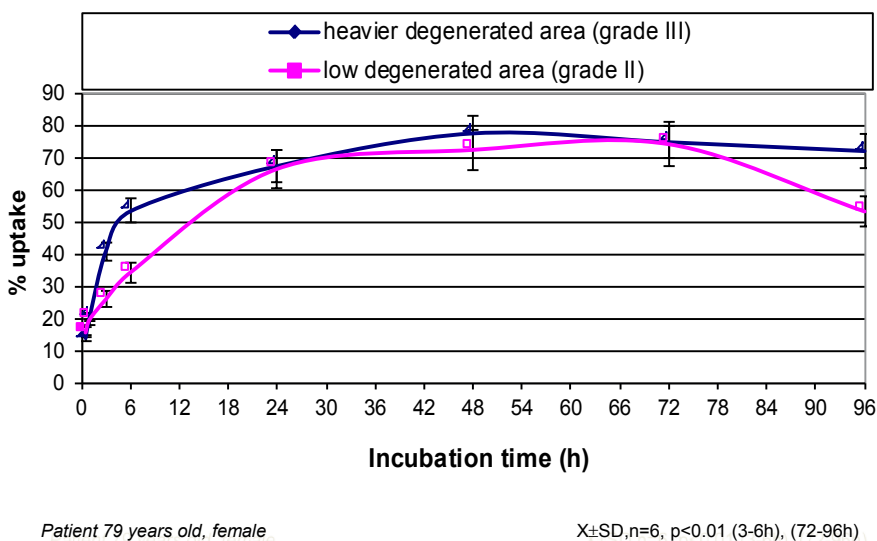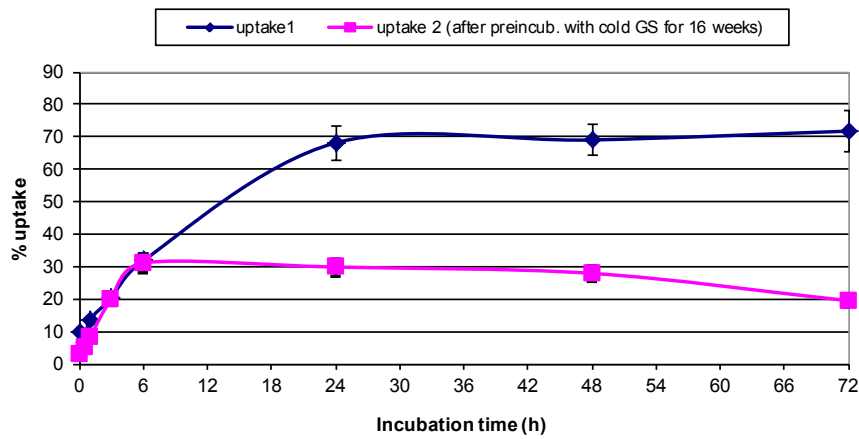

Figure 4: Uptake of [99m]TcGS in articular cartilage tissue pieces from the same patient but in areas of different grade of degeneration. The reduction of [99m]TcGS uptake was observed in low degenerated tissue areas (grade II) as compared to more severely degenerated tissue areas (grade III). Especially in the first period of incubation up to 6 h we observed significantly higher uptake of [99m]TcGS in heavier degenerated tissue areas.

Figure 5a: Uptake of 99mTcGS in articular cartilage tissue pieces (grade III) after preincubation with cold GS (80 µg/ml) for 16 weeks versus uptake with 99mTcGS alone. Preincubation results in lower uptake as compared to incubation with labeled 99mTcGS, proving the chondrotropic effects of GS during the treatment.

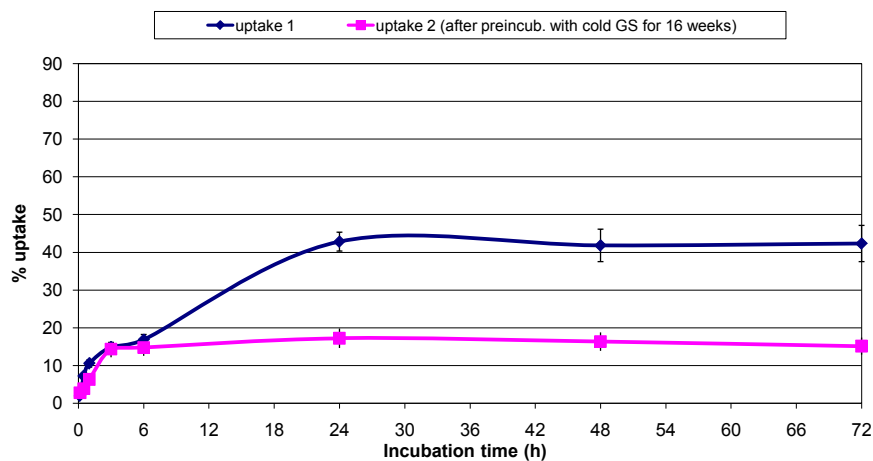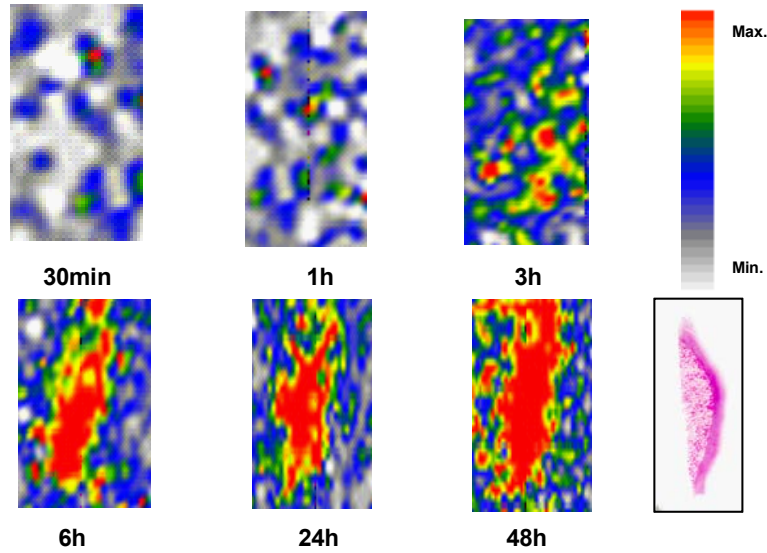**Figure 5b:** Uptake of 99mTcGS in articular cartilage tissue pieces (grade II) after preincubation with cold GS (80 µg/ml) for 16 weeks versus uptake with 99mTcGS alone. Preincubation results in lower uptake as compared to incubation with labeled 99mTcGS, proving the chondrotropic effects of GS during the treatment.

via NAc-GA or GA-6-phosphate into a nascent CS chain [24]. Not surprisingly, GA was found to stimulate PG production in human chondrocytes in vitro [28].

Labeling of GA (MW 180 D) is more difficult than that of larger polysaccharide entities such as CS(MW 25.000 D), since separation of the tracer from excess/free 99mTc may be hampered by the comparable MW of GS and the 99mTc label. Nevertheless, we achieved an acceptable degree of separation using molecular sieve chromatography on G10 gel, suggesting the formation of a tetravalent complex in which the central 99mTc4+ ion is surrounded by four GA molecules forming electron pair bridges via their amino groups (Figure 2). The molecular weight of the labeled 99mTcGS-complex was verified by molecular sieve chromatography and corresponds to the suggested structure (MW 1000 D).
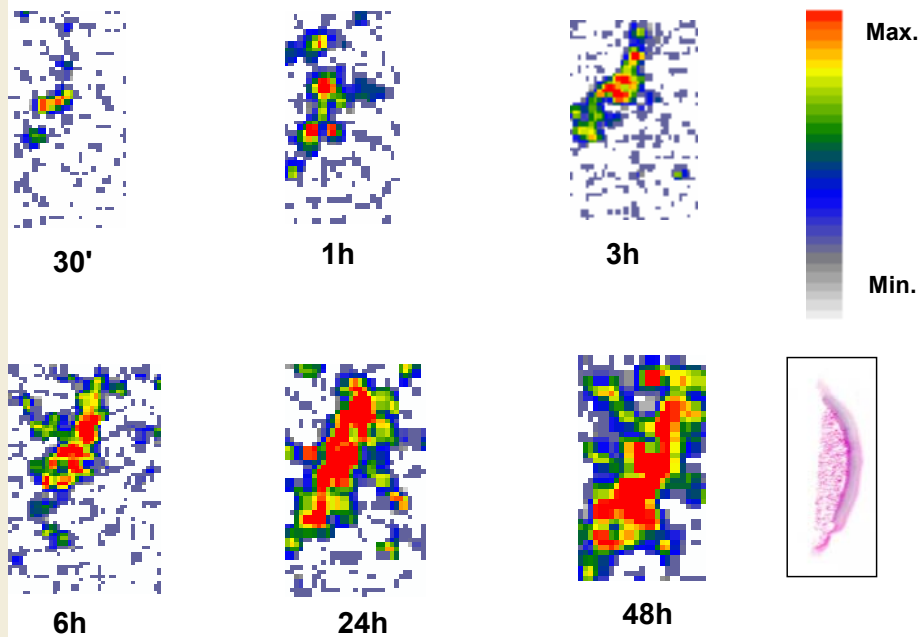
Autoradiography of articular cartilage with 99mTcGS

Figure 6: Autoradiographic uptake studies with ^{99m}TcGS (as an example) in degenerated human articular cartilage from patient with heavier degeneration (grade III). The dimensions of the autoradiographic images are 1.8:1 to the original size of the slices. The right side of individual slices represents the cartilage surface, left side the subchondral part of cartilage as shown in the histological image. We observed a continuously increasing uptake of the tracer during the whole incubation time.

Autoradiography with ^{99m}TcGS

Figure 7: Autoradiographic uptake studies with ^{99m}TcGS (as an example) in degenerated human articular cartilage from patient with lower degeneration (grade II). The dimensions of the autoradiographic images are 1.6:1 to the original size of the slices. The right side of individual slices represents the cartilage surface, left side the subchondral part of cartilage as shown in the histological image. We observed also a continuously increasing uptake of the tracer during the whole incubation time. The uptake in this patient tissue was lower as compared to the uptake seen in a tissue sample with heavier degeneration (grade III).

Autoradiography of articular cartilage with ^{99m}TcGS (non specific binding)

Figure 8: Non-specific uptake in degenerated human articular cartilage in the presence of 50-fold excess of cold GS in patients with grade III. The dimensions of the autoradiographic images are 1.8:1 to the original size of the slices. The right side of individual slices represents the cartilage surface, left side the subchondral part of cartilage.

As uniformly labeling of GA with ^{14}C or ^{3}H results in its metabolism we used instead a ^{99m}TcGS-complex. According to our results, free ^{99m}Tc liberated from such a complex does not significantly bind to cartilage (max 5% uptake). Only GA integrated in the ^{99m}TcGS complex is demonstrable since neither unbound GA nor its metabolized fragments are labeled. In addition, radiolabeling with a gamma emitting label such as ^{99m}Tc instead of ^{3}H or ^{14}C is necessary for prospective SPECT imaging studies in-vivo.

A clear indication for the significant in vitro stability of the ^{99m}TcGS-complex derives from the fact that it can be separated from free ^{99m}Tc by gel chromatography. For in vivo cartilage imaging significant complex stability should be granted over a time period of at least 2-6 h to avoid erroneous scintigraphic signaling from liberated free ^{99m}Tc. Another even more convincing proof for long-term stability of the ^{99m}TcGS-complex can be deduced from our finding that the complex in contrast to free ^{99m}Tc is strongly taken up by human cartilage in vitro, while the uptake of free ^{99m}Tc is negligible.

A further hint at long-term stability of our ^{99m}TcGS-complex comes from the results of preincubation experiments. When tissues were preincubated with cold GS, uptake of ^{99m}TcGS was strongly reduced. This finding proves that binding of complex-bound ^{99m}Tc was inhibited (Figures 5a and 5b). Quality control experiments using ITLC chromatography furthermore clearly show that the ^{99m}Tc-GA complex does not significantly dissociate over a time period of at least 6 h.

Both ^{99m}TcCS as well as ^{99m}TcGS can be used as scintigraphic probes for non-invasive diagnosis of early degenerative cartilage changes in OA, although each of the tracers acts via different pathways to delineate proteoglycan degradation. Charge effects, such as the reduced FCD, i.e. reduced concentration of negatively charged GAG-chains in degraded PG in OA cartilage cause both tracers to increasingly diffuse into the matrix. Katta et al. [29] recently showed that in vitro CS is able to diffuse into cartilage, the diffusion rate depending on the GAG content of the matrix. Our results provide the same evidence [30,31].

In contrast to CS, GA much more rapidly diffuses into the cartilage matrix because of its much smaller molecular weight (roughly 1.000 vs. 25.000). This fact results in faster diffusion of the tracer into the tissue, as compared to ^{99m}TcCS. We have demonstrated that the maximal uptake of ^{99m}TcCS was achieved in vitro after 24-48 h, while for ^{99m}TcGS between 6-24 h (depending on the degree of degeneration). This fact is important for the planned scintigraphic studies in patients suffering from OA.

While CS is able to bind to the cell surface of chondrocytes [32] via specific receptors, no such interaction is possible for GA. Nevertheless, GA is also taken up by the cartilage cells and integrated into GAG chains. The transport of GA through the plasma membrane is mediated by glucose transporter proteins [33]. Chondrocytes express several glucose transporters and actively take up and metabolize GA in contrast to N-acetyl-GA. There is an active competition between glucose and GA, GA inhibiting glucose transport in a non-competitive mode [34]. However, for both tracers, the contribution of cellular participation to their uptake by cartilage is limited, due to the low population density of chondrocytes in cartilage. That the overall uptake of ^{99m}TcGS is mediated via specific charged sites in cartilage is evident. Thus, even at saturation only 10% tracer is bound to the

cartilage in the presence of a 50 fold excess of cold GS.

In contrast to ^{99m}Tc-CS, however, the ^{99m}Tc-GS-complex is positively charged. Therefore, a higher uptake of the complex by heavily degenerated cartilage, as shown by us in the present paper, cannot be due to the decrease in FCD of this cartilage, as seen for ^{99m}TcCS, which is characterised by an overall negative charge of the molecule. Instead, the above mentioned repair mechanism combined with an increased leakiness of the pathologically altered tissue towards a relatively small molecular complex, might explain the fact that heavily degraded cartilage binds more ^{99m}TcGS than healthy or less degraded cartilage. In early OA i.e. before the onset of radiologic abnormalities, a significant decrease in uptake of GA is to be expected, however, as shown by others using other cationic cartilage targeting compounds such as ^{99m}Tc-NTP15-5 in a rabbit model of experimentally induced OA [35-37]. These tracers and related compounds are cartilage targeting, but not endogenous components of cartilage such as our ^{99m}TcGS.

We show that the in vitro uptake of ^{99m}TcGS depends on the degree of cartilage degeneration, i.e. PG depletion of the matrix. By autoradiographic uptake studies we have seen higher uptake in tissue at heavier degeneration (grade III) as compared to uptake of tissue with lower extent of degeneration (Figures 6 and 7). But not only uptake intensity was monitored. Also, the time curve of max. uptake (saturation) was correlated with degeneration and whereas at grade III the saturation was attained after 6 h of incubation, in contrast at grade II the saturation onset was observed only at 24 h.

Conclusion

Uptake of ^{99m}TcGS in degenerated cartilage in OA is tissue specific and correlates with tissue degeneration. Therefore, we believe that ^{99m}TcGS could be a suitable tracer to quantify degeneration in articular cartilage, due to its high uptake and sensitivity. As we found that uptake is degeneration dependent, we hope to take clinical advantage for early diagnosis of OA. In addition it could be a promising tracer to monitor therapeutic effects in OA.

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