EXTENDED REPORT

β Irradiation decreases collagen type II synthesis and increases nitric oxide production and cell death in articular chondrocytes

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Background: When synovitis is proved, intra-articularly injected β emitting radionuclides like yttrium-90 (^{90}Y) are used to treat the inflamed synovium.
Objective: To study the viability, matrix production, and NO production during or after ^{90}Y treatment of chondrocytes.
Methods: Monolayer, alginate, and explant cultures of primary bovine articular chondrocytes as well as synoviocytes were incubated with 0–3 MBq ^{90}Y/ml medium for four days from culture day 3 onwards. Cell viability was demonstrated by light and electron microscopy or by trypan blue or ethidium bromide/fluorescein diacetate staining, membrane integrity by measurement of lactate dehydrogenase (LDH) activity in the culture supernatants. Biosynthetic activity was demonstrated by incorporation of [^{3}H]proline and immunocytochemical staining of collagen type II. NO production was measured with the Griess reagent.
Results: In chondrocyte and synoviocyte monolayer cultures radiation caused a dose dependent increase in cell death and membrane destruction within four days. In alginate and explant cultures, where proliferation is low, no significantly increased LDH activity was seen, and cell viability was ~100% for up to 14 days after irradiation. Collagen type II expression (alginate) and biosynthetic activity (alginate and explants) were decreased dose dependently while there was an increase in NO production. Light and electron microscopy data showed that five weeks after irradiation all cells in alginate and most cells in explants subjected to 3 MBq ^{90}Y/ml were dead, whereas after lower amounts of irradiation several morphologically intact cells were found.
Conclusions: β Irradiation may influence the long term maintenance of cartilage tissue or the aetiology of degenerative joint diseases.

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Radiosynoviorthesis (RSYN) is a nuclear medicine treatment of inflammatory rheumatic and degenerative joint diseases. A β emitting radionuclide with a high radiant energy, a short half life (2.7 days for ^{90}Y), and a short range (mean range in tissues: 3.6 mm) is applied intra-articularly.[1] The radionuclide being phagocytised by the synovial lining cells leads to a locally high accumulation of radioactivity, which causes fibrosis and sclerosis of the inflamed synovial membrane,[2–4] thus improving the typical symptoms such as pain, a restricted range of motion, and relapsing joint effusions. A significant leakage of the radionuclide through blood vessels and lymphatic vessels is prevented by the colloidal preparation of the nuclide combined with a strict resting phase of the treated joint for at least 48 hours.[5 6] The synovial membrane, which is supposed to regenerate, should leave the patients free of symptoms for about a year. Thus, there is a temporary relief in symptoms but the progression of disease is not significantly affected by RSYN.[7] In comparison with surgical synovectomy, RSYN is easier to perform, costs less, and does not require any rehabilitation after the procedure.[5] RSYN is becoming more and more popular in Europe. However, the discussion about whether the treatment provides long term benefit for the patients becomes more and more controversial,[8–15] although most studies describe a positive outcome.[3 4 9 16]

RSYN was becoming established in the late 1960s but yet little is known about the influence of the radionuclide on other tissues of the target joint, like cartilage.[17 18] Important for our study was that the results of RSYN are most promising when the patients are young or the cartilage of the target joint is still relatively intact.[15 19 20] This makes it more important to determine whether ^{90}Y also affects cartilage. Therefore we developed a cell culture model simulating RSYN by irradiating synoviocytes and chondrocytes in a monolayer culture, and chondrocytes in a three dimensional alginate culture system and in tissue explants. After ^{90}Y incubation we examined the morphological appearance, cell viability, ^{90}Y toxicity, biosynthetic activity (collagen type II), and nitric oxide (NO) production of the cells. Alterations of these variables might also occur as side effects in vivo, which might influence the indication of RSYN treatment.

MATERIAL AND METHODS

Isolation and cultivation of bovine synoviocytes

Bovine synovial membrane was obtained from elbow joints of adult cattle at a local slaughterhouse under aseptic conditions. The synovial membrane was diced into 1–2 cm^2 pieces, washed in Hanks's balanced salt solution (HBSS, containing 100 U/ml penicillin/streptomycin and 2.5 µg/ml amphotericin; Biochrom, Berlin, Germany), and cultivated in 100 cm^2 culture flasks with Ham's F-10 (Biochrom) containing 10%

Abbreviations: ANOVA, analysis of variance; ETBr, ethidium bromide; FCS, fetal calf serum; FDA, fluorescein diacetate; HBSS, Hanks's balanced salt solution; LDH, lactate dehydrogenase; NO, nitric oxide; PBS, phosphate buffered saline; RSYN, radiosynoviorthesis; TBS, Tris buffered saline

fetal calf serum (FCS), 100 U/ml penicillin/streptomycin, and 2.5 μg/ml amphotericin (Biochrom), vitamin C (50 μg/ml, Sigma) and vitamin E (50 mM; Sigma T–3251). The culture medium was changed every third day (0.25 ml/cm^2). The synovial cells grew out of the tissue forming a monolayer on the bottom of the culture flasks. After one week the tissue pieces were discarded and the remaining synovial cells kept in a monolayer culture and passaged three times. For experiments the synovial cells were subcultured and seeded in a density of 25 000 synoviocytes/cm^2 in uncoated wells (Costar); passage numbers 4–6 were used for the experiments.

Isolation and cultivation of bovine articular chondrocytes

Bovine articular chondrocytes were isolated by a method described elsewhere.[21] In brief, cartilage was obtained from the articulatio tarsi transversa of adult cattle at a local slaughterhouse. Cartilage samples were washed in HBSS (containing 100 U/ml penicillin/streptomycin and 2.5 μg/ml amphotericin; Biochrom, Berlin, Germany) and diced aseptically into small fragments. These were treated with a mixture of pronase (32 U/ml; Boehringer Mannheim, Germany) and hyaluronidase (2.3 kU/ml; Sigma, Deisenhofen, Germany) in HBSS for one hour and then washed with HBSS containing antibiotics (see above for concentrations). Extracellular collagen was digested by incubation in a collagenase solution (collagenase (918 U/ml; Sigma, Germany) in Ham's F-10 (Biochrom, Germany) with vitamin C (50 μg/ml; Sigma), vitamin E (50 μM; Sigma), and 100 U/ml penicillin/streptomycin and 2.5 μg/ml amphotericin; Biochrom) for 6–8 hours. The resulting cell suspension was centrifuged (10 minutes at 400 *g*), the supernatant discarded, and the pellet resuspended in culture medium (Ham's F-10 containing 10% FCS (Biochrom), antibiotics (100 U/ml penicillin/streptomycin and 2.5 μg/ml amphotericin; Biochrom), and vitamins C and E (see above for concentrations)). The cell suspension was filtered through a gauze filter (mesh size 20 μm; Hydrobios, Germany) to obtain a single cell suspension. Viable cells were counted in a Neubauer chamber by the trypan blue exclusion method. They were seeded as primary chondrocytes in monolayer culture with a density of 100 000 cells/cm^2. Culture medium (0.25 ml/cm^2) was changed every third day.

Chondrocytes in alginate beads

Primary chondrocytes were isolated as described above. After determining the number of viable cells, the cell suspension was centrifuged, the supernatant discarded, and the pellet was resuspended and seeded in alginate (1.2% sodium-alginate from Fluka, Deisenhofen, Germany, in 0.9% saline solution; 10^7cells/ml alginate). The alginate beads were made by dropping the cell/alginate suspension through a fine injection needle (0.9 mm diameter) into a $CaCl_2$ suspension (102 mM). The beads (3 beads/well in a 12 well plate) were transferred into culture medium (see above). The medium (0.25 ml/cm^2) was changed every third day.

Isolation and cultivation of articular cartilage explants

Articular cartilage full thickness tissue (about 1 mm thickness) was isolated with a scalpel from the articulatio tarsi transversa of adult cattle at a local slaughterhouse. Cartilage samples were washed in HBSS (containing 100 U/ml penicillin/streptomycin and 2.5 μg/ml amphotericin; Biochrom, Berlin, Germany) and diced aseptically into explants of about 4×4 mm size. The explants were cultured in culture medium (Ham's F-10 containing 10% FCS; Biochrom), antibiotics (100 U/ml penicillin/streptomycin and 2.5 μg/ml amphotericin; Biochrom), and vitamins C and E (see above for concentrations), which was changed every third day. Three explants/well in a 12 well plate were cultured (0.25 ml medium/cm^2).

Irradiation

The culture medium (see above) was supplemented with 0–3 MBq ^{90}Y/ml medium (CIS bio International, Cedex, France) on culture day 3. On day 7 the medium was discarded and either the different tests were performed or the cells, beads, and explants were washed (3×5 minutes each) with buffer and kept in culture with ^{90}Y-free culture medium for long term examinations.

Cell morphology of monolayer cultures

On culture day 7 the cells were washed with HBSS and fixed with 4% paraformaldehyde. The culture plates were stored at 4℃ for the next three weeks for radioactive decay. After three weeks the monolayer cultures were assessed under a phase contrast microscope.

Cell viability in alginate beads and cartilage explants

Cell viability was assessed by a method described elsewhere for cartilage explants.[22] In brief, after irradiation, the alginate beads and tissue explants were sectioned with a scalpel blade into slices of about 100 μm thickness. Three to four slices of three explants/group were stained, each with 10 μl of a dye solution containing 5 μg/ml ethidium bromide (EtBr) and 50 μg/ml fluorescein diacetate (FDA) in phosphate buffered saline (PBS). EtBr only enters cells which have lost cell membrane integrity. EtBr interacts with DNA and forms a red fluorescent complex. FDA readily crosses the cell membrane and is then enzymatically converted by intracellular esterases into a green fluorescent anionic product, which can no longer leave the cell. Fluorescence was visualised using a fluorescence microscope (Ex 450–490 nm), and dead or alive cells were counted. About 100 cells/section were counted. Living cells were given as a percentage of total.

^{90}Y toxicity (release of lactate dehydrogenase (LDH))

The toxicity of ^{90}Y was determined by measuring the LDH activity using a cytotoxicity detection kit (catalogue No 1 644 793, Boehringer Mannheim, Germany). Chondrocytes, synoviocytes, and tissue explants were cultivated and irradiated as described above. After irradiation the culture supernatants were centrifuged (five minutes, 1000 *g*) and 100 μl used as samples. To determine LDH activity in the alginate beads the beads were incubated with 1 ml dissolving buffer (55 mM sodium citrate, 50 mM NaCl) and the solution was centrifuged (five minutes, 1000 *g*). 100 μl of the supernatant was used as the sample. The LDH activity in the supernatants and in the beads of the alginate cultures was then added to give a total LDH release. For all types of culture the basic outflow of LDH activity during the four day incubation in non-irradiated controls was evaluated and the mean value was subtracted from all other sample values as the background level. Parallel samples of each yttrium group were incubated after the four day irradiation with 1% triton X-100 for five hours to measure the total LDH content of the cells. These values were added to the mean outflow of each group and were used as a group-specific 100% of LDH (total). LDH release was given as a percentage of the total.

Histology of alginate beads and cartilage explants

Alginate beads were fixed by treatment for 30 minutes with 4% paraformaldehyde with 10 mM $CaCl_2$ and then washed several times with cacodylate buffer (0.1 M, pH 7.4, 50 mM $BaCl_2$ and eosine 0.005%) until the Ca^{2+} precipitate

disappeared. Tissue explants were fixed with paraformaldehyde alone and washed with PBS. Then the beads and explants were dehydrated with rising concentrations of isopropanol (75%, 96%, and 100%), transferred into xylol, and embedded in paraffin. Thin sections (7 µm) were made using a sled-microtome (Leitz, Wetzlar, Germany) and spread out on microscope slides coated with 4.5% (wt/vol) gelatine solution containing 300 mM potassium chrome sulphate-12-hydrate.

Immunocytochemical staining of collagen type II

The paraffin sections of alginate beads were deparaffinated with xylol and transferred into aqua dest. using decreasing concentrations of ethanol (100%, 96%, and 75%). Then they were treated with a 0.1% pepsin solution (3.9 kU/ml in 0.5% acetic acid; Sigma) for 30 minutes and rinsed with Tris buffered saline (TBS; 0.14 M NaCl in 20 mM Tris/HCl buffer, pH 7.4). Unspecific peroxidase was blocked by treatment with 0.6% H_2O_2 in methanol for 20 minutes. The samples were rinsed again with TBS and treated with the primary antibody (clone CII 1b, DSHB, Iowa City, USA, diluted 1:10 with TBS) directed against collagen type II for one hour. After rinsing with TBS, the samples were treated with the second antibody (rabbit antimouse IgG, horseradish peroxidase conjugated, Dako P0260, Hamburg, Germany, diluted 1:200 with TBS containing 1% bovine serum) for 30 minutes, rinsed again with TBS, and incubated with the third antibody (goat antirabbit IgG, horseradish peroxidase conjugated, Dako P0448, diluted 1:100 with TBS containing 1% bovine serum) for 30 minutes. Before staining with diaminobenzidine (DAB Kit, Vector Laboratories, Burlingame, USA), the samples were again rinsed with TBS. Cell nuclei were counterstained with Meyer's haemalum (Merck, Darmstadt, Germany), diluted 1:1 with distilled water, and the stained samples were mounted using Aquatex (Merck).

[^{3}H]proline incorporation

Alginate beads and tissue explants were incubated for six hours in culture medium (see above) supplemented with [^{3}H]proline (10 µCi/ml; Amersham Pharmacia). The beads were washed in PBS containing 1 mM Ca^{2+}, and the explants in PBS alone. Each bead and explant was digested separately in 1 ml PBS (pH 6.5) containing 0.1 M Na_2HPO_4, 10 mM Na_2-EDTA, and 0.125 mg/ml papain (2.125 U/ml; Sigma) at 65℃ overnight. On the next day 200 µl of each digested sample was added to 2 ml of scintillation fluid (Hydroluma, Baker Chemicals), mixed, and measured with a scintillation counter (Wallac 1904, Turku, Finland). The digestion mixture (500 µl) was mixed with 500 µl PBS and used to determine the DNA content as described below.

Measurement of DNA

Samples (see proline incorporation) and blanks (containing only 1 ml PBS) were treated for 15 seconds with an ultrasonic beam (sonopuls gm 70, Bandelin, Berlin, Germany). Then 0.5 ml of RNAse (5 kU/ml) and 0.5 ml of pronase (0.7 U/ml) were added and the mixture was incubated for 30 minutes at 37℃. After this, 0.5 ml of EtBr (25 µg/ml) was added, and the samples were measured with a fluorometer (Hitachi F2000; $\lambda_{ex} = 365$ nm; $\lambda_{em} = 590$ nm) after 30 minutes' incubation. DNA content was computed from a standard curve.

Nitric oxide production

Generation of nitric oxide was determined by measuring nitrite accumulation in culture supernatants, culture media (w/o cells), and human synovial fluid (synovia) using Griess reagent (1% sulphanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5% H_3PO_4; Sigma). Sample and Griess reagent (100 µl of each) were mixed and incubated for five minutes, the absorption was estimated by an automated plate reader (SLT Reader 340 ATTC) at 540 nm. Sodium nitrite ($NaNO_2$, Merck, Darmstadt, Germany) was used to generate a standard curve for quantification.

Electron microscopy

For electron microscopy the alginate cultures were fixed for 30 minutes with 3% glutaraldehyde in PBS (containing 10 mM $CaCl_2$), and the tissue explants with glutaraldehyde without $CaCl_2$. The alginate beads were washed using cacodylate buffer (0.1 M+50 mM $BaCl_2$; 3×10 minutes each), and the tissue explants were washed using PBS alone. All samples were incubated (2×15 minutes each) with 1% OsO_4 (Pasel und Lorei GmbH; Frankfurt, Germany) and 1.5% potassium ferrocyanide (Merck, Darmstadt, Germany). Samples were dehydrated with ascending concentrations of isopropanol, embedded in araldite, and ultrathin sections were prepared. The sections were incubated for 15 minutes with saturated uranylic acetate in 70% methanol, hydrated in descending concentrations of methanol, and incubated in a plumbic citrate solution under a CO_2-poor atmosphere for five minutes. The sections were viewed with an electron microscope (Zeiss 900, Germany). Three explants/group were sectioned. Three sections/explant were visualised, and about 50 cells/section were evaluated for morphological appearance.

Statistics

All biochemical data are presented as mean values (SD). The significance of differences between irradiated groups and non-irradiated controls was calculated using the two tailed Student's *t* test and in some cases differences among groups of different concentrations of ^{90}Y were tested with a one way analysis of variance (ANOVA).

RESULTS

After four days of ^{90}Y irradiation an alteration in cell viability and morphology in synoviocyte and chondrocyte monolayer cultures was seen. There was a decrease in cell numbers and the remaining cells were visibly damaged in comparison with control cultures where the cells still appeared to be intact (light microscopy data not shown). In accordance with the morphological studies, ^{90}Y irradiation with concentrations higher than 0.1 MBq/ml ^{90}Y reduced the cell membrane integrity in synoviocyte and chondrocyte monolayer cultures significantly (two tailed Student's *t* test; $p<0.05$). The release of LDH was increased after four days of irradiation and varied in synoviocyte cultures between 68 (8.6) (0.375 MBq), 84 (21.3) (1.0 MBq), and 73 (16.1) (3.0 MBq)% of the total activity in the supernatants, and in chondrocyte cultures between 67 (3.5) (0.375 MBq), 55 (6.3) (1.0 MBq), and 63 (5.8) (3.0 MBq)% of the total activity related to non-irradiated controls (~0%). LDH activity in the supernatants of cultures irradiated with 0.1 MBq/ml ^{90}Y was 2.5 (2.1)% in synoviocyte cultures and 3.1 (2.4)% in chondrocyte cultures (% of total, n = 3; mean (SD)). However, in three dimensional alginate cultures and in cartilage tissue explant cultures the membrane integrity of chondrocytes was not significantly changed even by higher doses of ^{90}Y after four days of irradiation, indicating an intact cell membrane of the cells: alginate cultures 1.35 (2.3) (0.375 MBq), 0 (3.5) (1.0 MBq), and 1.76 (2.6) (3.0 MBq)% of the total activity (mean values (SD); n = 6) and explant cultures: 0.9 (3.7) (0.375 MBq), 2.2 (4.4) (1.0 MBq), and 2.4 (3.4) (3.0 MBq)% of the total activity (mean values (SD); n = 6).

Cell viability of the chondrocytes in alginate and explant cultures was still >95% on culture day 21 (two weeks after ^{90}Y irradiation; in all irradiated and non-irradiated groups)

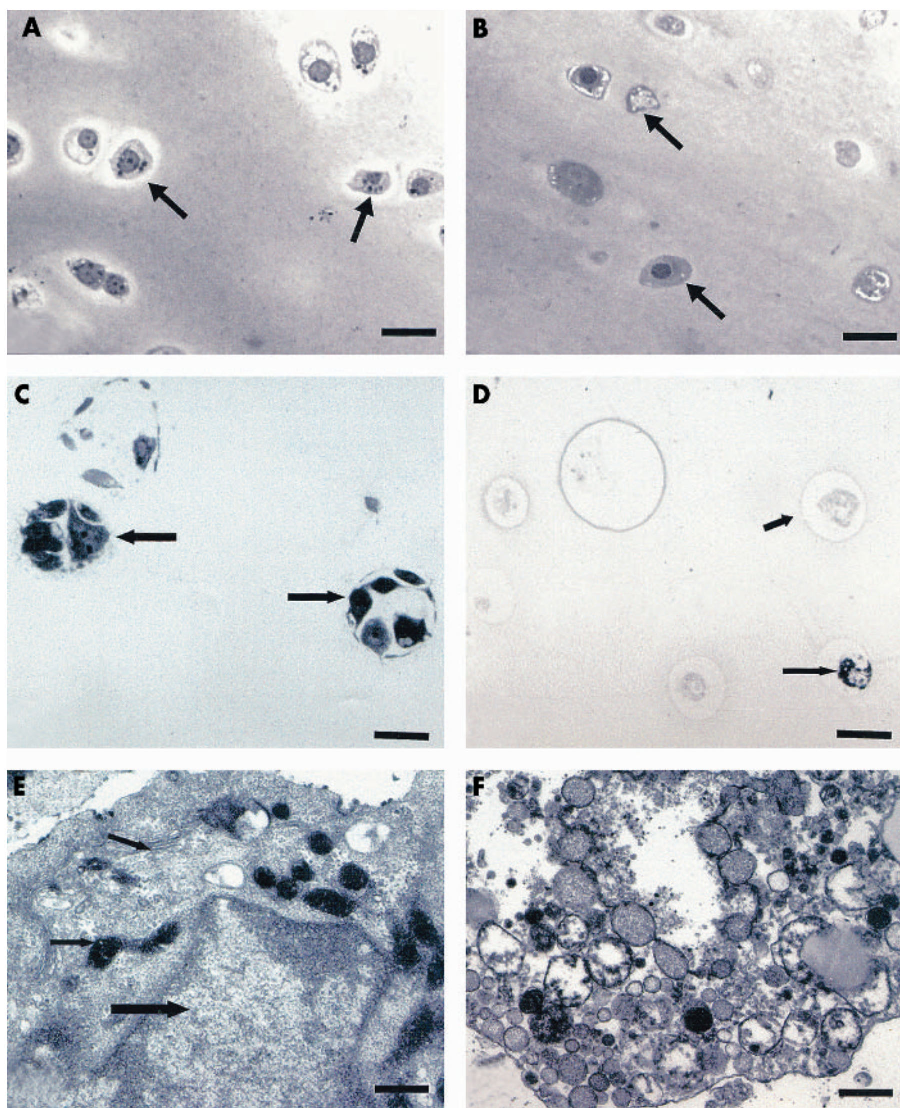

Figure 1 Semi-thin sections (A-D) and electron microscopy (E, F) of cartilage explants (A, B) and primary chondrocytes in alginate (C-F) five weeks after a four day period of β irradiation indicating an induction of cell destruction. (A, C and E) Control cultures. Arrows demonstrate intact cells in A and C or intact organelles like the nucleus (large arrow) and the golgi apparatus and mitochondria (small arrows) in E. (B, D, and F) 3 MBq ^{90}Y/ml. Arrows demonstrate damaged cells in B and D. In F remnants of organelles of a dead cell are visible. (A-D) bar = 27 μm, (E, F) bar = 0.2 μm.

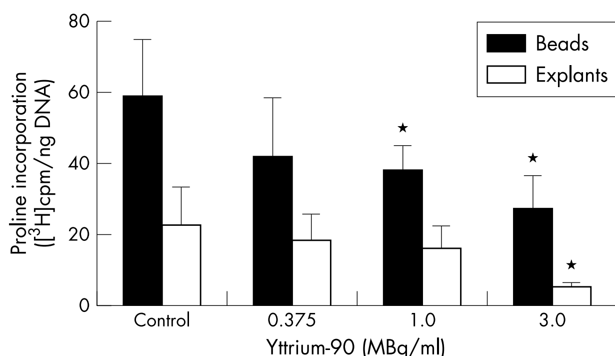

Figure 2 [^{3}H]Proline incorporation in chondrocyte alginate cultures and cartilage explants two weeks after a four day period of ^{90}Y irradiation. The bars show the dose dependent decrease of [^{3}H]proline incorporation ($p<0.004$ by one way ANOVA) related to non-irradiated control cultures. Mean values (SD); n = 6 (explants n = 3); *$p<0.05$ *v* control by two tailed Student's *t* test.

demonstrated by EtBr or FDA staining. Nevertheless, light and electron microscopy data showed that five weeks after irradiation with 3 MBq/ml all cells were morphologically damaged (fig 1). At lower ^{90}Y concentrations most of the cells still looked intact, indicating a dose dependent long term effect of ^{90}Y irradiation on cell viability of chondrocytes in alginate and tissue explants. Cells with or without lower ^{90}Y irradiation (<3 MBq/ml) formed clusters of cells predominantly at the edge of the alginate beads. However, cultures irradiated with 3 MBq ^{90}Y formed no cell clusters at all.

Protein synthesis of chondrocytes in alginate and explant cultures was decreased dose dependently by ^{90}Y irradiation (fig 2). [^{3}H]Proline incorporation demonstrated that protein biosynthesis of the cells was still significantly influenced two weeks after ^{90}Y irradiation. The biosynthetic activity was decreased by ^{90}Y dose dependently to ~46% in alginate and 22.6% in explant cultures (3 MBq/ml) in comparison with control cultures. Collagen type II production in alginate cultures was demonstrated two weeks after irradiation (fig 3). Immunohistochemical staining showed a dose dependent

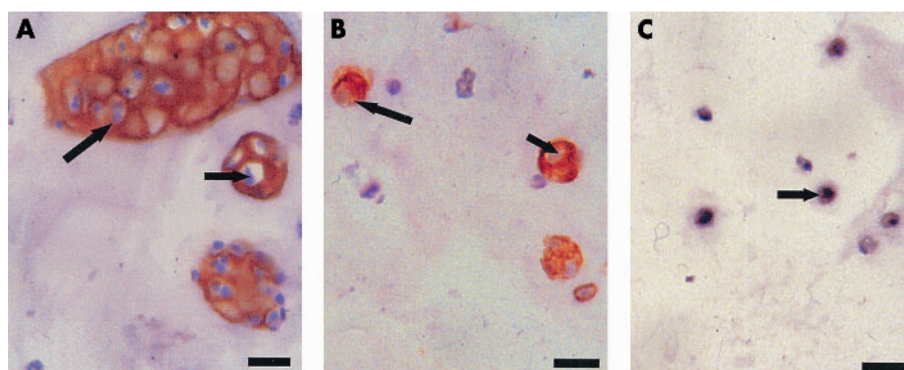

Figure 3 Immunocytochemical staining of collagen type II on cultures of chondrocytes in alginate beads two weeks after a four day period of ^{90}Y irradiation. The figure shows the dose dependent decrease of collagen type II expression (brown staining; arrows demonstrate cells) compared with (A) the non-irradiated control; (B) 0.375 MBq; (C) 1 MBq ^{90}Y. Bar = 15.38 μm

decrease in pericellular collagen type II deposition by ^{90}Y while there was a strong staining for collagen type II around cells in non-irradiated cultures.

NO production was determined in culture supernatants of alginate and explant cultures after four days of irradiation (fig 4). The data showed a significant dose dependent increase in accumulation of the NO end product nitrite with rising concentrations of ^{90}Y activity (ANOVA). Comparable results were found after irradiation of cell-free culture medium as well as samples of human synovial fluid (synovia), where ^{90}Y also induced a dose dependent production of NO (fig 4).

DISCUSSION

Because RSYN is even more effective when the bone and cartilage tissues of the target joint are still relatively intact or the patients are young,[15 19 20] it is important to point out that the effects of β irradiation on cartilage tissue have not been fully investigated.[17 18] Therefore we developed a cell culture system which represents an in vitro model of RSYN to examine the effects of ^{90}Y on chondrocytes.

We made several calculations about the concentration of yttrium in our experiments. The relation between the ^{90}Y activity used for each defined volume of cartilage tissue in vivo can only be estimated approximately. For an idealised globe structure the volume of one single chondrocyte would be about 7.2×10^{-6} mm^3. Eckstein *et al* found that the mean volume of the cartilage in a human knee joint is about 23.245 mm^3 ±19%.[23] The fraction of the cells in cartilage tissue is estimated as about 5%. Therefore the mean volume of chondrocytes in the whole cartilage of a human knee joint would be 1.162 mm^3 and, consequently, the total number of cells 1.6×10^8. This is the number of chondrocytes that can potentially be affected by the irradiation with 185 MBq ^{90}Y performing RSYN in a human knee joint. Three alginate beads/culture well with a mean number of 1.5×10^5 chondrocytes/alginate bead were used, and thus the ^{90}Y activities used for our studies have to be calculated for 4.5×10^5 chondrocytes. 185 MBq ^{90}Y/1.6×10^8 chondrocytes in vivo corresponds to ~0.5 MBq ^{90}Y/4.5×10^5 chondrocytes in vitro. Therefore the activities of ^{90}Y (0–3 MBq) used in vitro can be considered as comparable with the conditions in vivo. Additionally, considering a total cartilage surface of 120 cm^2 (Eckstein *et al*)[23] there would be a relation of 1.5 MBq ^{90}Y/cm^2 surface in vivo. The alginate beads used in our studies have a mean total surface of ~0.9 cm^2. Performing the experiments with ^{90}Y activities between 0.375 and 3 MBq, we also found comparable conditions between the in vivo and the in vitro situation. Finally, we did previous tests to ensure that the lowest concentration of ^{90}Y would still have the effect on synoviocytes which is seen in clinical studies (cell death and morphological changes of the obviously damaged cell monolayer).

Our data show that there is a dose dependent long term effect of ^{90}Y irradiation on cell viability of chondrocytes in the three dimensional alginate and tissue explant culture system. In contrast, there is an immediate and striking influence on cell viability in both types of monolayer cultures (chondrocytes and synoviocytes). Chondrocytes that are kept in monolayer culture dedifferentiate after a few days, which means that they are starting to proliferate. Additionally, they stop producing chondrocyte-typical extracellular matrix components (collagen type II). The alginate matrix and the native

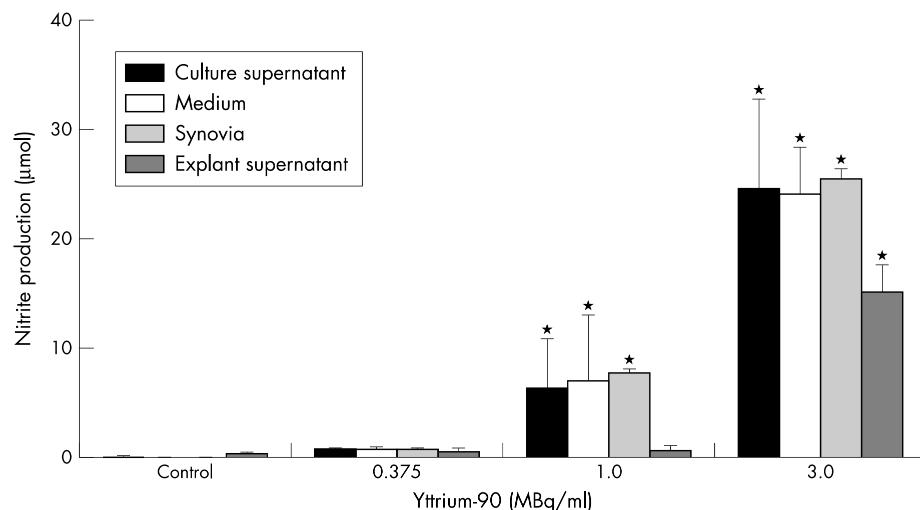

Figure 4 NO accumulation in supernatants of seven day old chondrocyte alginate and explant cultures, human synovial fluid (synovia) samples, and cell-free culture medium during four days of ^{90}Y irradiation. The bars show the dose dependent increase of NO accumulation in the different systems. Mean values (SD); n = 6 (explants n = 3); *$p<0.001$ *v* control by two tailed Student's *t* test; $p<0.001$ by one way ANOVA.

matrix in explants provides an in vivo-like surrounding for chondrocytes, which prevents the cells from dedifferentiation and proliferation.[24] One might speculate that this restriction of cell division—which is normal for chondrocytes in vivo—is the reason why irradiation damaged the DNA and why, subsequently, chondrocyte viability is delayed in comparison with monolayer cultures. β Irradiation is also known to induce cell death (apoptosis) in various cell types or genetic damage through DNA strand breaks.[25–27] In our studies cell death in monolayer cultures might be caused by some sort of DNA strand breaks, because cell death was induced much more quickly in proliferating cell culture systems. Nevertheless, it might be speculated that some of the cell death that we found in our study might be related to NO induced apoptosis,[28] because NO is known to induce apoptosis in chondrocytes under certain circumstances.[29] This has to be investigated in further studies.

Strand breaks in chondrocytes of alginate and explant cultures might also influence the process of transcription and, subsequently, the maintenance of cell metabolism-like production of extracellular matrix in the long term. Thus, we determined the cellular activity of the cells in alginate beads and explant cultures after irradiation by measuring [^{3}H]proline incorporation and, more specifically, collagen type II expression in alginate cultures. We found that [^{3}H]proline incorporation and collagen type II expression were dose dependently decreased after irradiation in comparison with non-irradiated controls. Three mechanisms may be responsible for these effects. Firstly, it is well known that β irradiation can cause genetic damage through DNA strand breaks,[27]in various cell types. Those might become apparent in the process of transcription and subsequently in the production of extracellular matrix. Our methods cannot explain whether the production of collagen type II is reduced by these mechanisms or whether the damaged cells produce defective collagen type II which can no longer be detected by our immunocytochemical staining method. However, it is most likely that the collagen type II production is decreased because the cellular activity measured by [^{3}H]proline incorporation was also decreased after β irradiation.

Secondly, ^{90}Y induced dose dependent generation of NO was found in vitro and even in human synovial fluid (synovia). The increased production of NO in cell cultures, medium, or synovial fluid might be explained by the fact that exposure to ionising radiation causes radiolysis of water leading to the generation of reactive oxygen species.[29] Possibly, also, molecules containing nitrogen, like arginine, might be split by ionising radiation. Hashimoto *et al* and others found that NO reduces collagen type II expression in cartilage tissue.[28 30–32] Thus, possibly, there is a connection between the ^{90}Y induced NO production and the decrease in collagen type II synthesis that we found in our studies.

Finally, reduced biosynthetic activity might be a consequence of increasing cell death in the different culture systems, although the cells still had intact membranes two weeks after irradiation. This raises the question of whether cells that appear to be alive by FDA staining might still be in the process of dying.

Further investigations are needed to show whether surviving cells after low dose irradiation have the potential to recover and return to normal cellular activities. The integrity of chondrocytes with a functional biosynthetic activity is very important for long term maintenance of cartilage tissue. The knee cartilage, especially, has to withstand large mechanical forces and for this requires complete and intact tissue. Therefore it might be speculated that patients develop gonarthrosis much earlier because of decreased cell viability, restricted biosynthetic activity, and consequently less extracellular matrix production in the cartilage tissue of the affected joint. To our knowledge no studies have yet reported induction of osteoarthritis by early RSYN. This should be investigated further. If RSYN induces long term reduction of collagen type II expression or chondrocyte viability its use in young patients with intact cartilage tissue should be carefully discussed.

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