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EFFECTS OF IONIZING RADIATION ON THE SIZE DISTRIBUTION OF PROTEOGLYCAN AGGREGATES SYNTHESIZED BY CHONDROCYTES IN AGAROSE

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Abstract

Effects of ionizing radiation on the structure and size-distribution of newly synthesized proteoglycan aggregates are studied in-vitro. Chondrocytes, isolated from embryonic chick sterna, are cultured for 7 days in a tri-dimensional agarose system. Single radiation doses of 10, 20 and 50 Gy are given before starting the culture. Digestion of the artificial agarose matrix liberates the newly synthesized proteoglycans. Spreading with cytochrome C allows electron microscopic investigations of the individual, newly synthesized molecules.

The structure of aggregates synthesized by control and irradiated chondrocytes is comparable. However, radiation causes alterations in the size-distributions of the aggregate-populations. For the control cultures, an average aggregate contains 27 aggrecans per aggregate. 34 pro mille of the molecules contain more than 100 aggrecans per aggregate. Irradiation with 10 Gy doesn't cause alterations. With radiation doses of 20 and 50 Gy, an average molecule contains 20 aggrecans. Only about 9 pro mille of the aggregates contain more than 100 aggrecans. Stimulation of lysosomal activity after irradiation could explain the observed alterations.

Key Words : Ionizing radiation, agarose culture, proteoglycan aggregates, embryonic cartilage.

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Introduction

Although the main effect of irradiation is related to inhibition of cell division, there are additional acute effects related to metabolic disturbances. Cartilage and extracellular matrix are known to be relatively radioresistant. However, in previous studies, we observed that single radiation doses caused a dose-dependent decrease of incorporation of radio-labeled collagen- and proteoglycan-precursors, already within a 7 days post-irradiation period (Cornelissen et al., 1990a, 1993a). In these studies, doses ranging from 20 to 100 Gy were applied, covering the dose range used radiotherapeutically e.g. for the treatment of larynx carcinoma (50-60 Gy). Although an effect of radiation was observed on the synthesis of the matrix components by these incorporation studies, no morphological differences on the ultrastructure of the matrix components (collagen fibers and proteoglycans) were observed (Cornelissen et al., 1990b, 1993a). The reason for this could be the following. First of all, in these experiments, we used cultures of cartilage fragments. So we observed extracellular material composed of components synthesized before as well as after irradiation. Secondly, using a fixation method to visualize proteoglycans on semi-thin sections (by adding a cationic dye to the fixative), proteoglycan molecules appear as electron dense granules, which appearance does not correlate with their structure in-situ. However, when proteoglycan aggregates are isolated and spread by the Kleinschmidt technique, they have an extended 'brushlike' structure, which is compatible with biochemical data (Hascall, 1980).

In this study, we want to investigate the effect of ionizing radiation on the structure of neosynthesized, native proteoglycan aggregates. For this reason, cell cultures of chondrocytes are performed in a three-dimensional agarose system. The particular way to liberate the proteoglycans enables us to look at whole populations of de novo synthesized molecules.

Materials and Methods

Isolation procedure

Chondrocytes were isolated from embryonic chick sterna (stage 41 Hamburger and Hamilton, 1951). The method described by Verbruggen et al. (1990) was used with a few modifications. Briefly: for each experiment, 20 sterna were sliced into small pieces (1 mm^3). The chondrocytes were liberated from the cartilage matrix by sequential treatment with hyaluronidase (0.2 %, 60 minutes, 37°C), pronase (0.2 %, 60 minutes, 37°C) and collagenase (0.2 %, 60-90 minutes, 37°C). Isolated cells were washed twice in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % foetal calf serum (FCS) and antibiotics. After isolation, usually more than 90 % of the cells were viable (Trypan blue test).

Irradiation procedure

The isolated chondrocytes were irradiated with a single ration dose of 10, 20 or 50 Gy at a dose rate of 1 Gy per minute. Irradiation was performed with a Philips MG 420 X-ray system, operating at 250 kV constant potential with an additional filtration of 1 mm Al and 1.1 mm Cu, corresponding to a radiation quality with a measured HVL of 2.1 mm Cu. Chondrocytes were irradiated in 1 ml DMEM.

Agarose cultures

After irradiation, chondrocytes were washed in DMEM. Cultures of irradiated and control chondrocytes were performed as follows. Tissue culture plates (Costar) were coated with 3 % agarose (Type I-A, Sigma). The artificial matrix was formed by mixing 3 % agarose with an equal volume of double concentrated DMEM supplemented with 20 % FCS and 100 µg/ml ascorbate. The isolated chondrocytes were added to this 1.5 % agarose solution in the smallest possible volume. A final density of 1.5 x 10^6 cells per culture (2 ml) was used. After gelification of the agarose (30 minutes at 4°C), 1 ml of DMEM with 10 % FCS, antibiotics and 50 µg/ml of freshly dissolved ascorbate was added as nutrient medium. Cultures were kept at 37°C with 5 % CO_2 for 7 days. Nutrient medium was replaced twice.

Preparation of cultures for light microscopy

At the end of the culture period (7 days), parts of the gel containing the cells are fixed in glutaraldehyde (2.5 % in 0.1 M cacodylate buffer, pH 7), supplemented with 1 % Alcian blue to precipitate the proteoglycans (Schofield et al., 1975). After embedding in epoxy resin, semi-thin (1 µ) sections were stained with Alcian blue (1 % in 3 % acetic acid, pH 2.5) to demonstrate the proteoglycans.

Liberation and visualisation of the proteoglycan molecules

Proteoglycan (PG) molecules were obtained by digestion of the artificial agarose matrix with agarase (50 U/ml of phosphate buffered saline, overnight at 40°C). The resulting suspension was centrifuged for 10 minutes at 100 g to pellet the cells. The Kleinschmidt protein layer technique was used to visualize the PG (Kleinschmidt and Zahn, 1959). 10 µl of the supernatant containing the molecules was mixed with 10 µl buffer (0.5 M Tris HCL, 0.05 M EDTA : pH 8.5), 10 µl cytochrome C (2 mg/ml), 10 µl 6 M NH_4-acetate and 10 µl aq. dest. This solution was released on the surface of 0.25 M NH_4-acetate. The PG-cytochrome C film was picked up with nitrocellulose coated grids. They were immediately immersed in uranylacetate (5 x 10^{-2} M uranylacetate in 5 x 10^{-2} M HCl) for 1 minute and rotatory shadowed with platinum-palladium at an angle of 7°. Electron microscopy was performed with a Jeol 1200 EXII microscope.

For each radiation dose, 3 different cultures were observed. For each culture, 3 x 100 molecules on 3 different grids were counted.

Results

Light microscopy of the cell cultures

Pictures of control and irradiated chondrocytes, cultured for 7 days in agarose, are given in Figure 1. Alcian blue positive material, indicative for the presence of PG, is present in the irradiated cultures as well as in the control cultures. In each culture, about 95 % of the cells show an Alcian blue positive extracellular border (arrows)

Structure of PG-aggregates synthesized after irradiation

The structure of the PG-aggregates synthesized after irradiation is comparable to the structure of aggregates synthesized by the control, non-irradiated chondrocytes (Figure 2). They consist of a central hyaluronan chain to which several aggrecans (PG-monomers) are attached. In irradiated as well as in control cultures, the number of free aggrecans is abundant. No empty hyaluronan chains nor gaps on the chain are observed.

Size distribution of the synthesized PG-aggregate populations

Histograms showing the size distribution of the PG-aggregates in the studied molecule populations are given in Figure 3. Control cultures show a majority of aggregates consisting of 5 to 50 aggrecans per aggregate. An average aggregate contains 27 aggrecans per aggregate. However, an important amount of very large molecules is found. Characteristics of the synthesized aggregates are given in Table 1. 34 pro mille of the molecules contains more than 100 aggrecans per aggregate. Chondrocytes irradiated with 10 Gy show no marked differences with the controls. The same size distribution was found, with the same amount of very large molecules. However, differences are observed in the cultures irradiated with 20 and 50 Gy. The number of large molecules is decreased markedly. In both, the 20 and 50 Gy cultures, only 9 pro mille of the molecules consists of more than 100 aggrecans per aggregate.

Discussion

The use of chondrocytes cultured in 1.5 % agarose, digestion of the artificial agarose matrix with agarase and spreading of the synthe-

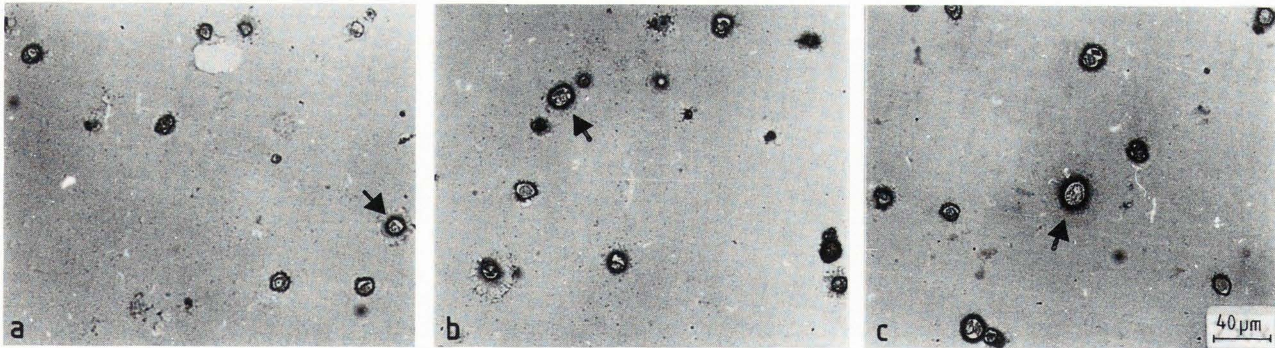

Figure 1. Micrograph of chondrocytes cultured in agarose for 7 days. a: control, b: 20 Gy, c: 50 Gy. Alcian blue positive material, indicative for the presence of proteoglycans, is present around the cells (arrows). Fixation : glutaraldehyde supplemented with Alcian Blue. Staining : Alcian blue.

Table 1 : Characteristics of aggrecan-aggregate populations, synthesized after different radiation doses by embryonic chick chondrocytes in agarose cultures. The average number of aggrecans per aggregate (x), the range in the population and the amount of aggregates containing more than 50 and 100 aggrecans (pro mille) are given. For each experiment, 900 aggregates are studied.

	Control	10 Gy	20 Gy	50Gy
x	27	28	21	50
range	5-317	5-284	5-135	5-233
> 100	34	33	9	9
> 50	130	124	69	61

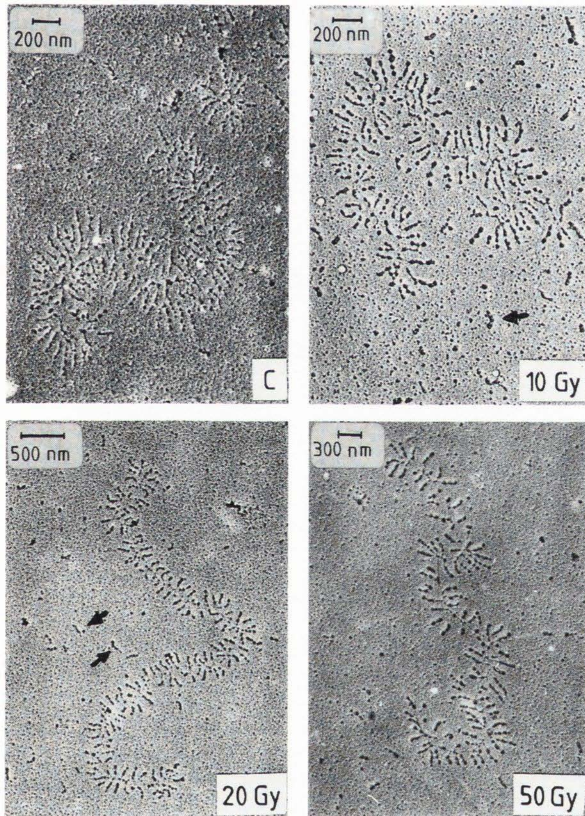

Figure 2. Electronmicrograph of proteoglycan aggregates, synthesized by embryonic chick chondrocytes in agarose during a 7 days post-irradiation period. Aggregates consist of a central hyaluronan chain to which several aggrecans are attached. A large number of free aggrecans (arrows) are present. Spreading : Kleinschmidt technique, staining : uranyl acetate, shadowing : platinum-palladium.

syzed PG molecules by the Kleinschmidt technique enable us not only to observe the structure of native, de novo synthesized molecules, but also create the possibility to observe almost the whole population of synthesized aggregates.

In previous work, we showed that agarase digestion of the artificial agarose matrix does not affect the structure of the synthesized molecules (Cornelissen et al., 1993b). The structure of the PG-aggregates synthesized in-vitro in agarose and liberated by agarase digestion of the artificial matrix is comparable to structures described in-vivo (Buckwalter and Rosenberg, 1988) and in-vitro (Kimura et al., 1978). As already known, a proteoglycan aggregate is composed of a central unbranched hyaluronan chain to which several PG-monomers are attached. A direct correlation exists between the length of the hyaluronan chain and the number of attached monomers. So the number of monomers per aggregate is a measure of aggregate size (Buckwalter and Rosenberg, 1988). The use of agarose in a concentration of 1.5 % immobilizes almost all PG-aggregates and digestion of the artificial agarose matrix liberates about 90 % of the synthesized proteoglycans (Verbruggen et al., 1990). So the agarose culture system combined with the spreading technique is not only suitable to describe aggregate structure but also permits size measurements of the synthesized aggregate population.

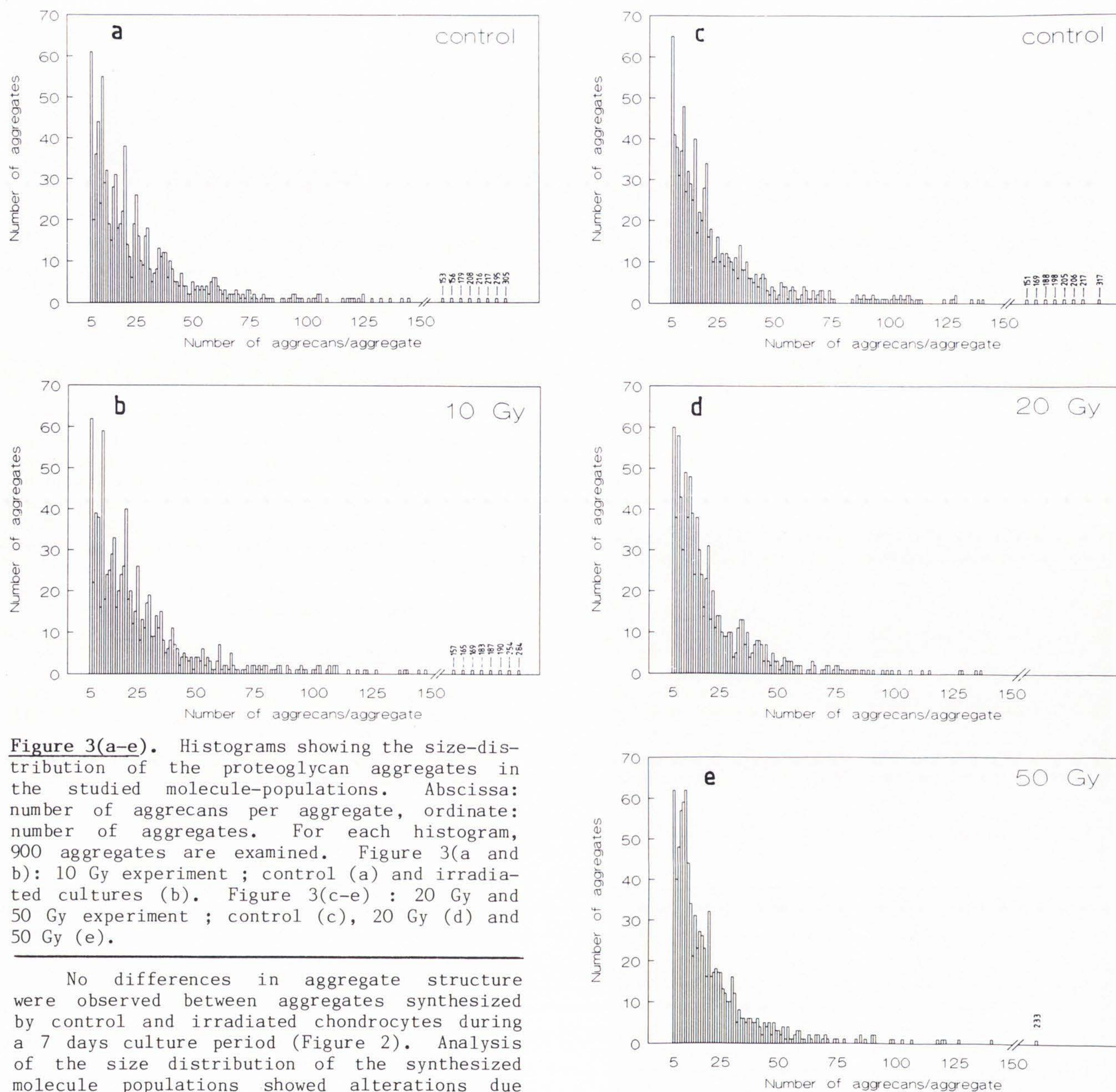

Figure 3(a-e). Histograms showing the size-distribution of the proteoglycan aggregates in the studied molecule-populations. Abscissa: number of aggrecans per aggregate, ordinate: number of aggregates. For each histogram, 900 aggregates are examined. Figure 3(a and b): 10 Gy experiment ; control (a) and irradiated cultures (b). Figure 3(c-e) : 20 Gy and 50 Gy experiment ; control (c), 20 Gy (d) and 50 Gy (e).

No differences in aggregate structure were observed between aggregates synthesized by control and irradiated chondrocytes during a 7 days culture period (Figure 2). Analysis of the size distribution of the synthesized molecule populations showed alterations due to irradiation starting from a radiation dose of 20 Gy. Differences were mainly found in the small fraction of very large aggregates (Table 1). Aggregates containing more than 100 monomers were almost absent in cultures of chondrocytes, irradiated with 20 or 50 Gy (9 pro mille instead of 34 pro mille for the controls). A radiation dose of 10 Gy on the other hand caused no alterations in the aggregate population. Even after this high radiation dose, chondrocytes synthesized PG-aggregates that are, concerning size and structure, comparable to those synthesized by non-irradiated cells. Since PG synthesis is a rather complex process combining formation of both protein and glycan, the continuation of synthesis of 'normal' macromolecules indicates the inherent insensitivity of chondrocytes to ionizing radiation.

The absence of large aggregates after irradiation with 20 and 50 Gy indicates fragmentation of the long hyaluronan chains. The question remains if fragmentation is the result of synthesis of shorter chains or due to degradation after synthesis of 'normal' chains. The latter seems most likely. Also in physiological and pathological conditions, characterized by a decrease in aggregate size, it is accepted that degradation occurs extracellularly after synthesis of normal chains. Different possible mechanisms are mentioned. Under pathological conditions, for example in arthritic cartilage, fragmentation of the aggregate is described to be generated by a non-enzymic mechanism (reactive oxygen metabolites e.g. H_2O_2) (Roberts et al., 1987). Degradation of the hyaluronan

chain in the presence of radical-generating systems has also been described by others (Greenwald and Moy, 1980). H_2O_2 as the agent of degradation of aggregates seems unlikely in our radiation experiments since generation of H_2O_2 only occurs during the short period of irradiation. In our experiments, radiation occurs on isolated chondrocytes, in the absence of extracellular material so that only newly synthesized PG-aggregates were studied. Also under physiological conditions, e.g. during the process of enchondral ossification, aggregate fragmentation is described (Buckwalter, 1983). According to Lash and Vasan (1983) the agent of degradation could be lysosomal. Since we found in previous radiation experiments on cartilage (Cornelissen et al., 1990b) an increase of acid phosphatase, a marker of lysosomal activity, after irradiation of cartilage, stimulation of lysosomal enzymes could lie on the basis of the observed alterations.

We concluded that an ionizing radiation dose of 10 Gy caused no alterations in the newly synthesized PG-aggregate-population during a 7 days post-irradiation period. After an administration of 20 or 50 Gy, large aggregates, containing more than 100 monomers, were almost absent. Stimulation of lysosomal activity could be responsible for the observed alterations.

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Discussion with Reviewers

T.D. Allen : The authors are suggesting a stimulation in acid phosphatase activity as a potential cause for the observed radiation effect. Is the lysosomes acid phosphatase going to be membrane acid phosphatase and might this affect the size of the PGs in the ECM?
Authors : In the previous article, mentioning the dose- and time-dependent increase of acid phosphatase activity (AP) after ionizing radiation, a semi-quantitative morphological method was used. The number of AP positive cells and the amount of AP in the cells was correlated with the radiation dose but also dependent on the post-irradiation time. After 7 days of culture, precipitates of AP positive material were not only found in the cells, but also in the surrounding extracellular matrix. So we believe that stimulation of AP could have an effect on the aggregates, present in the matrix.

K. Goltry : Agarose cultures were analysed after 7 days. Why 7 days ? Is this time period necessary for the assay ?
Authors : Analysis of the agarose cultures was performed after 7 days because of quantitative reasons. We wanted to look at early radiation effects, but the use of the cytochrome

C spreading technique asks for a certain amount of molecules. Experimentally we find out that in our system a period of 7 days was sufficient to obtain enough molecules.

K. Goltry : You say there are an important number of larger aggregates present in the control cultures. Is there a biological significance for these larger aggregates ? Do you know of any functions specific versus smaller aggregates ? Do you believe the loss of larger aggregates plays a role in the condition you mentioned in the discussion (i.e. enchondral osification, arthritis), or are they just a side effect of some increased lysosomal activity?

Authors : It is known that certain metabolic processes or pathological phenomena are accompanied or characterized by alterations in the size of the proteoglycan aggregates. An important characteristic of large aggregates is their inhibitory effect on matrix calcification. We believe that alterations in the size of the aggregates indeed play a role in enchondral ossification and that the loss of their inhibitory power is due to alterations in their size. A more general biological significance for very large aggregates vs. smaller ones is that it is assumed that these large molecular complexes are involved in the growth and repair of tissues.

K. Goltry : What other types of proteoglycans do chondrocytes synthesize ? Can you study them using this agarose system ?

Authors : A cartilage proteoglycan is composed of a core protein to which keratan-sulfate and chondroitin-sulfate are attached in different regions. This structure is called an aggrecan or proteoglycan monomer. Typical for cartilage is the presence of aggregates, formed by monomers attached to hyaluronan. The keratan-sulfate rich region is near the binding site with hyluronan. Changes can occur in the relative amount of keratan- and chondroitin-sulfate. These phenomena can be studied using the technique described in the paper, using some slight modifications. For example the use of carbon reinforced grids instead of nitrocellulose coated grids makes it possible to distinguish between the side-chains on the core protein.

K. Goltry : Have you tried to examine the glycan content of the proteoglycan aggregates or look for any changes in levels of sulfation before and after irradiation ? Would this be possible or relevant, in your agarose system ?

Authors : In this agarose system we didn't look for changes in levels of sulfation before and after irradiation. The aim of the work was to look at the ultrastructure and size distribution of the newly synthesized aggregates. However, it would be possible and interesting to perform incorporation studies with $Na_2{}^{35}SO_4$ and ^3H-glucosamine. The latter will be incorporated in the glycosaminoglycans of both, the proteoglycans and hyaluronan ; the first will predominantly be incorporated in the proteoglycans. If the incorporation is followed by a separation technique (CsCl-density-gradient centrifugation, electrophoresis, column chromatography), also an idea about the incorporation in the different macromolecular fractions can be obtained.

V.C. Hascall : It is stated that the amount of Alcian blue positive material in the different cultures were comparable between irradiated and control. How was this determined ? A simple cell count/viability assessment combined with a quantitative dye binding assay for PG concentration on the released cell matrix would provide the necessary data to support or dispute these statements.

Authors : The presence of Alcian blue around the cells was only checked light microscopically to be sure if synthesis of proteoglycans had occured before starting the rather time comsuming procedure of preparation of the molecules for electron microscopic investigation.

J. Wroblewski : Our own studies have shown that low doses of irradiation (10-16 Gy) lead to inhibition of proliferation in cultured epithelial cells. Have you noticed any differences in the number of cells or the total amount of synthesized PG after varying doses of irradiation ?

Authors :In this paper we did no quantification of DNA or proteoglycan synthesis. However, in earlier studies (de Ridder et al., Int. J. Rad. Biol., 1988 and Cornelissen et al., Int. J. Rad. Biol., 1990) we examined the effect of ionizing radiation on both DNA and proteoglycan synthesis by incorporation of radio-labeled thymidine and glucosamine. Thymidine incorporation was already decreased with 50 % after administration of 10 Gy, while incorporation of glucosamine was only halfed after administration of 100 Gy (both after 3 days of culture).