Acta orthop. Scandinav. 39, 58–72, 1968

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IN VITRO INCORPORATION OF S^{35}-SULFATE IN CHONDROSARCOMATOUS TISSUE

By

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Received 3.iv.67

INTRODUCTION

Radical surgery is, at present, the only appropriate form of therapy for chondrosarcoma (*Dahlin & Henderson* 1956). Non-radical surgery leads to relapse and radiotherapy is without significant effect. Since chondrosarcomas are most often localized in the proximal part of the femur, in the pelvis, or in the proximal part of the humerus, surgery must be performed as disarticulation, hemipelvectomy, or forequarter amputation. The results seem to justify such measures: a 50 per cent 15-year cure rate has been reported for chondrosarsomas of the extremities (*Hakelius & Hjelmstedt* 1964), and a 30 per cent 10-year cure rate after hemipelvectomy for chondrosarcoma of the pelvis (*Troup & Bickel* 1960).

While radical surgery is thus relatively successful, it obviously creates new problems for the patient. Hence efforts must be made to obviate the necessity of major amputations, through the development of new forms of treatment, which, in turn, may require the elaboration of new diagnostic methods. However, a more comprehensive knowledge of the biochemistry and metabolism of chondrosarcoma is a prerequisite condition for such attempts.

Financial support was obtained from the Swedish Medical Research Council, King Gustav V 80th Birthday Fund, the Knut and Alice Wallenberg Foundation and the Association for the Aid of Crippled Children, New York. A preliminary account of this work was given at the Annual Meeting of the Swedish Orthopaedic Association, Nov. 25–27, 1965, Stockholm, Sweden.

A characteristic feature of chondrosarcomata is the formation of an abundant matrix, which, as in normal cartilage, contains sulfated glycosaminoglycans[1]. The biosynthesis of such compounds has been studied intensively in recent years; thus, the various enzymes and precursors involved are known in some detail, as reviewed elsewhere (*Boström & Rodén* 1965).

From a therapeutic standpoint, there are here two methods of attack. First, an attempt to retard the growth of a chondrosarcoma by interfering with the formation or maintenance of its matrix. For instance, several substances are known to have an inhibitory effect on the biosynthesis of sulfated glycosaminoglycans, *viz.* cortisone, salicylates, phenylbutazones, and anti-malarials (*Boström, Berntsen & Whitehouse* 1964). Furthermore, it has been shown that parenteral administration of papain can cause the breakdown of the ground substance of preformed, cartilaginous matrices (*Thomas* 1956).

Second, the exploitation of the pronounced anabolic activity of the tumor cells in order to introduce a precursor, which, when built into the sulfated glycosaminoglycans, would cause damage to these cells. This approach was used by *Gottschalk et al.* (1959), *Gottschalk* (1960), *Andrews et al.* (1960), and *Botstein & Marcus* (1963), who administered S^{35}-sulfate to patients with advanced chondrosarcoma for internal irradiation of the tumor tissue. The radio-isotope was given in amounts of 0.6–1.2 C. With this dosage, temporary arrest of growth and relief of pain was noted in several cases. That high doses of S^{35}-sulfate have a definite radiotoxic effect on growing cartilage is evident from studies on rats (*Rubin et al.* 1957) and on mice (*Gottschalk & Beers* 1958).

These therapeutic trials were based on tracer studies, which included autoradiography of biopsy material, and demonstrated a preferential uptake of the isotope by the tumor tissue. It was also established that the limiting factor in the therapeutic use of S^{35} is the radiosensitivity of the hematopoietic system. Marked depression of bone marrow activity invariably followed the administration of the large doses of S^{35} employed. However, the marrow function was completely restored in about a month. Consequently, the possibility of multiple courses of S^{35}-treatment for patients who respond well was suggested by *Botstein & Marcus* (1963), and is currently being explored in a case with lung metastases (*Boström et al.*, unpublished observations).

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[1] In this paper we have adopted the nomenclature used in The Amino Sugars (eds. E. A. Balazs & R. W. Jeanloz, Academic Press, New York 1965).

Apparently, the general application of isotope treatment of chondrosarcoma cannot, as yet, be warranted. Nonetheless, in advanced cases it is clearly justifiable to administer S^{35}-sulfate in order to further explore the therapeutic possibilities and/or to examine the metabolic activity of the tumors.

For the latter purpose clinical studies should preferably be extended to include *in vitro* experiments on surgically removed tumor material. This would allow the use of a variety of labelled precursors and would also permit an analysis of the effects of different chemical compounds on the metabolism and proliferation of the tumor cells. It is conceivable that in this way it may be possible to develop a method for the diagnostic and prognostic evaluation of chondrosarcomatous tumors.

The present investigation represents such an attempt to augment clinical trials with S^{35}-sulfate through laboratory experiments. It is, moreover, concerned with the correlation between the morphology of the tumor and its utilization of S^{35}sulfate *in vitro* as revealed by autoradiography, and also with the incorporation of the isotope into different fractions of glycosaminoglycans.

MATERIAL AND METHODS

The material was obtained from two cases of chondrosarcoma of a long bone in which amputations were performed.

Case 1. Boy, born 1952. At ten years of age pains in the left hip; roentgenograms showed cystic lesions in the trochanter major region (Figure 1). Curettage was performed. Histologic diagnosis: chondroma. Two years later the pains in the hip recurred, and roentgenograms showed progress of the cystic lesions. As biopsy established the diagnosis of chondrosarcoma, hemipelvectomy was performed. No postoperative complications.

Two years after the operation the patient was free from symptoms, and adapted himself satisfactorily to his prosthesis.—Presumably the tumor was a chondrosarcoma from the outset, but the histologic signs of malignancy were at first underestimated.

Case 2. Male, born 1918. When 45, felt pain in left shoulder; interpreted as cervical radiculopathy. Roentgenological examination was made only 9 months later, showing a destructive lesion in the proximal humerus (Figure 2). Biopsy established a diagnosis of chondrosarcoma. Since the tumor extruded into the soft tissues in the anterior part of the axilla, local resection could not be performed and, consequently, forequarter amputation was resorted to. Postoperative local necrosis required skin transplantation.

Eighteen months after operation the patient was in good general condition, with no signs of metastasis; he adapted himself well to his prosthesis.

Fiure 1. Case 1. Roentgenogram showing chondrosarcoma in the proximal part of the left femur.

Figure 2. Case 2. Roentgenogram showing chondrosarcoma of the proximal part of the left humerus extending into the soft parts of the axilla.

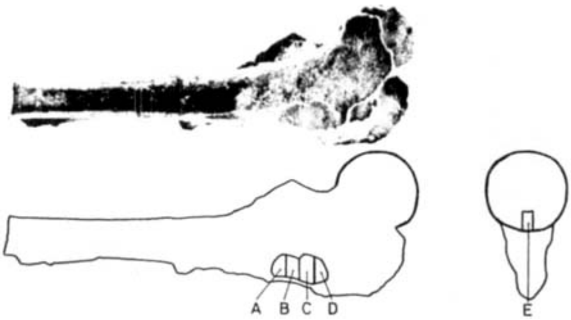
Sampling of Tumor Tissue

Each long bone was sawed in two longitudinally, and the cut surfaces photographed. One half of each bone was used for histologic diagnosis and radioisotope studies respectively. For these studies, tissue blocks were cut from the tumors *in situ*. The blocks measured approximately 5 × 5 × 5 mm and were taken from macroscopically different areas of the tumors, as indicated in Figures 3 and 4.

From the tumor in case 1 four blocks were taken, designated A–D; in case 2 three blocks were sampled (A–C). For control, blocks of articular cartilage were also taken from each bone and were marked E and D respectively.

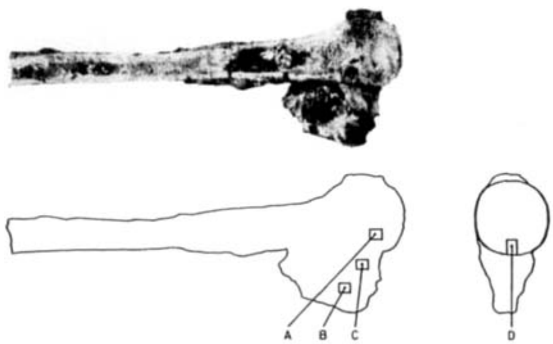
Incubation

Before incubation each tissue block was cut into two parts, one for biochemical investigation and the other for autoradiography. Each of these parts was subdivided into slices approximately 0.5 mm which were incubated for two hours in 5 ml of a Krebs-Ringer solution containing 100 μC of radioactive sulfate. After incubation the process was stopped by addition of 1 ml of 2 per cent monoiodoacetic acid. The slices were then carefully washed, with water and excess of unlabelled sodium sulfate, according to *Boström et al.* (1955).



	CPM / mg	% of Control
A	19800	769
B	23540	915
C	23870	927
D	40282	1565
E	2574	100

Figure 3. Case 1. Incorporation of S^{35}-sulfate (total glycosaminoglycan fraction) into various zones of the tumor (A-D) and into normal joint cartilage (E = control). Radioactivity expressed as counts per minute per mg dry tissue.



	CPM / mg	% of Control
A	1630	155
B	3884	370
C	9858	938
D	1051	100

Figure 4. Case 2. Incorporation of S^{35}-sulfate (total glycosaminoglycan fraction) into various zones of the tumor (A–C) and into normal joint cartilage (D = control). Radioactivity expressed as counts per minute per mg dry tissue.

Biochemical Analyses and Radio-Assays

The slices for examination were further washed in alcohol and ether and dried *in vacuo*, weighed on a Cahn micro-balance and digested with papain. Complete solubilization of the material was obtained.

From all the digested samples aliquots were taken for indirect determination of

S^{35}-sulfate incorporation into the total glycosaminoglycan fraction. The radiocativity of the aliquots was measured before and after precipitation with ethanol and subsequent centrifugation, the difference between the two measurements was regarded as representing the S^{35}-activity of the total glycosaminoglycan fraction.

The digested samples from case 1 were utilized also to evaluate the incorporation of the isotope into individual glycosaminoglycan fractions. These were obtained by the micro-separation technique of *Antonopoulos et al.* (1963); the radioactivity of each resulting fraction was measured.

Assays of S^{35} activity were made on material plated at infinite thinness. A Geiger-Müller counter with a 1.9 mg/cm^2 end window tube was used. Radioactivity was expressed as counts per minute per mg of dry tissue.

Morphology and Autoradiography

The incubated and washed slices were fixed in alcoholformalin (3 + 1 parts) and embedded in paraffin. Semi-serial sections were cut at 2 and 4 microns, and mounted on glass slides. About half the number of slides were used for staining and the others for autoradiography. The following staining procedures were applied: hematoxylin and eosin, astrablau (*Bloom & Kelly* 1960), and toluidine blue (0.1 per cent in 70 per cent ethanol).

Coated autoradiograms were prepared by a modification of the *Messier & Leblond* method (1957). Ilford K5 nuclear emulsion was used, diluted with either one part of water (4 micron sections) or four parts of water (2 micron sections). Exposures lasted from one to two days. The autoradiograms were developed in Kodak D 19b, cover-slipped, and were examined by light and by phase-contrast microscopy with qualitative evaluation of density. Quantification through grain counting was not attempted since the autoradiograms were too dense over the cells for this, in spite of the short exposure time, a rather high S^{35}-dosage being used for the *in vitro* incubations to ensure an adequate level of activity for the G.-M. counting, particularly in the fractionation study.

RESULTS

Biochemical analyses and radio-assays

Figures 3 and 4 show the incorporation of radioactive sulfate into the total glycosaminoglycan fraction of the various zones of the tumors and adjacent joint cartilage. These figures indicate that the uptake of S^{35}-sulfate was much higher in all tumor samples than in the corresponding samples of normal cartilage, but that there were also considerable variations between the samples of an individual tumor. Thus, the radioactivity of the tumor samples exceeded that of the control cartilage by a factor of about 7 to 15 times in case 1, and of about 1.5 to 9 times in case 2.

The results of the fractionation study made in case 1 are summarized in Figure 5. The variation in total radioactivity between the different

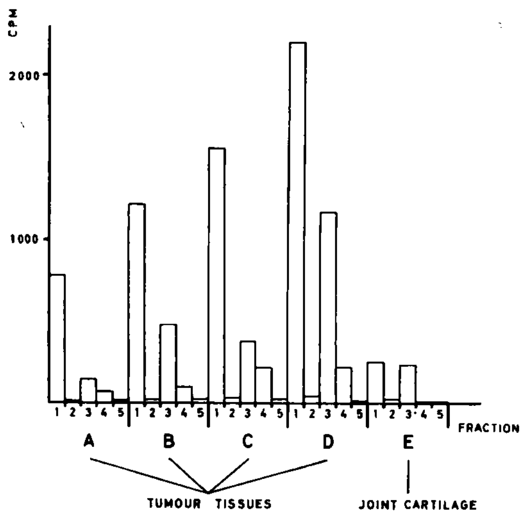


Figure 5. Case 1. Radioactivity in various glycosaminoglycan fractions in tumor tissue (A–D) and into normal joint cartilage (E). Fraction 1: keratan sulfate, fraction 2: hyaluronic acid, fractions 3 and 4: chondroitin sulfates, fraction 5: heparin-like compounds.

samples essentially parallels that shown in Figure 3. All the tumor samples show a similar distribution pattern of radioactivity between the five fractions. This pattern is distinctly different from that of normal joint cartilage, owing mainly to a predominance, by a factor of two to five, in the radioactivity of fraction 1 over that of fraction 3, but also to the occurrence of significant radioactivity in fraction 4.

Morphology and autoradiography

The histologic examination of the tumors showed that both were rather highly differentiated chondrosarcomas. However, their appearance varied markedly, from area to area, in respect of both cellularity and character of the matrix. For instance, zones with abundant matrix alternated with clusters of cells (Figure 6 A). The matrix was collagenous in some parts of the tumors and myxomatous in other parts. Formation of chondroid tissue was noted in case 2 (Figure 7 A). Necrotic areas were occasionally observed.

A direct relationship was readily evident between microscopic appearance and uptake of S^{35}-sulfate by the tumor samples, as determined by G.-M. counting. The more cellular and the more 'active' the appearance

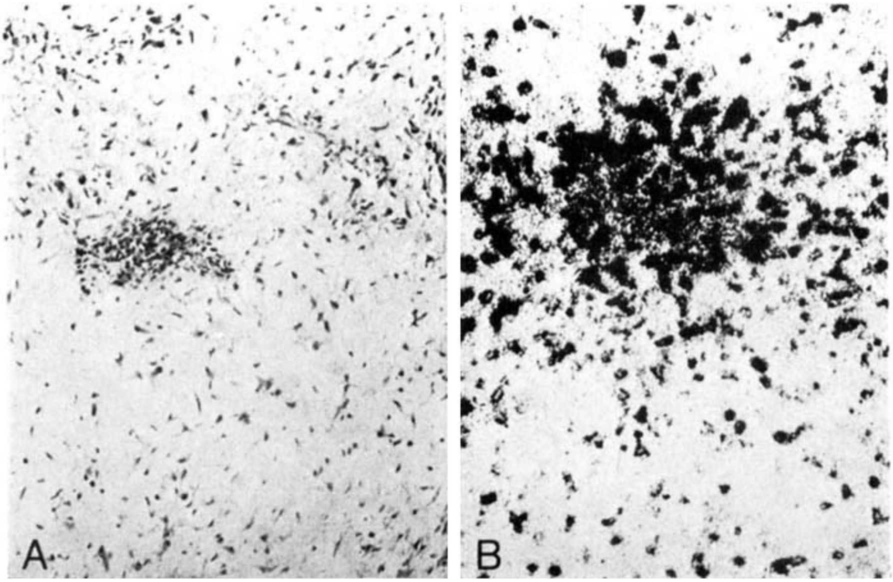


Figure 6. Case 1. A: section of the tumor stained with hematoxylin and eosin. B: coated autoradiogram showing the incorporation of S^{35}-sulfate into a similar area of the tumor. × *95.*

of the sample, the higher was its radioactivity. Of the two tumors, that in case 1 had the more malignant appearance and showed the most pronounced uptake of the isotope.

This correlation between morphology and isotope incorporation was directly visible in the autoradiograms. These show that, in both tumor tissue and control cartilage, the uptake of S^{35}-sulfate took place as a massive cellular incorporation (Figures 6B, 7B and 8). No uptake of the isotope occurred in necrotic tumor areas.

In the samples of joint cartilage there was no indication of a secretion of S^{35}-labelled matrix components by the chondrocytes. However, in the tumor samples, notably within the cell clusters in case 1, rather dense accumulations of silver grains were also located over the matrix between, or immediately surrounding the cells (Figure 6B).

DISCUSSION

An increased *in vitro* uptake of radioactive sulfate into the total glycosaminoglycan fraction by the chondrosarcomatous tissues, as compared with the uptake of the normal joint cartilage, was demonstrated in this

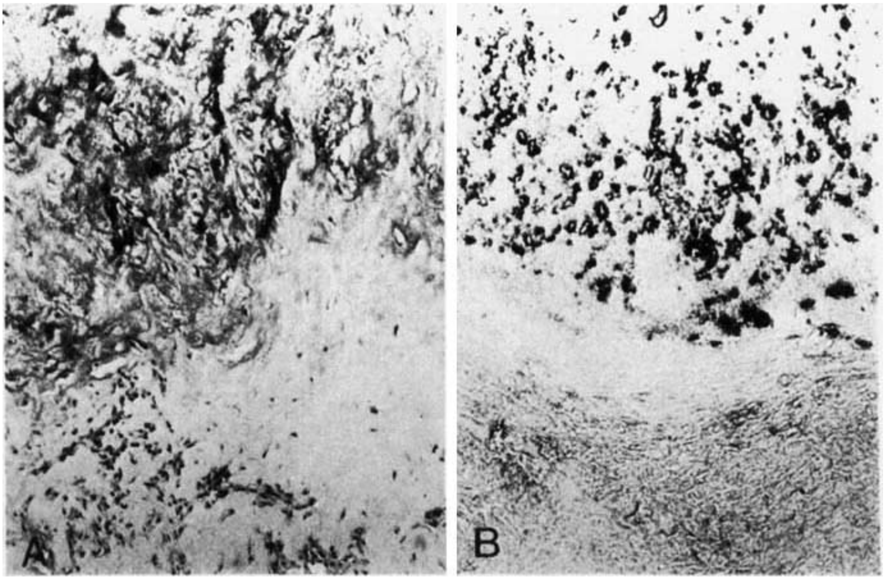


Figure 7. Case 2. A: section of the tumor stained with hematoxylin and eosin. B: coated autoradiogram showing the incorporation of S^{35}-sulfate into a similar area of the tumor. × *95.*

investigation. This is in good agreement with earlier observations *in vitro* (*Layton* 1949, *Wolfe & Vickery* 1964) and *in vivo* (*Gottschalk et al.* 1952, 1959, *Gottschalk* 1960, *Andrews et al.* 1960, *Botstein & Marcus* 1963).

The greater cellularity of the tumor tissues is a major factor affecting this increased uptake, as indicated by the morphologic and autoradiographic findings. Moreover, the variations in radioactivity between the different tumor samples were clearly related to cellular content. Conceivably, an accelerated turnover may also be involved, but this could not be demonstrated with certainty in the present material. Variations in synthetic rate among skeletal cells can, in general, be detected by autoradiography, namely through evaluation, preferably by grain counting, of the initial incorporation of the isotope into the cells, or through observation of the subsequent transfer rate of labelled components to the matrix (see, for instance, *Öberg* 1964). The latter procedure requires the harvesting of samples after consecutive time intervals, which was not possible in this study, because of the limited amount of material available; it has already been mentioned that grain counting was not feasible. Nonetheless, the occurrence of silver grains

Figure 8. Case 1. Coated autoradiogram showing the incorpation of S^{35}-sulfate into normal joint cartilage. × *95.*

over tumor matrix may be taken as indicating increased synthetic rate compared with that of normal cartilage. These silver deposits may be interpreted as representing a secretion of labelled glycosaminoglycans into the intercellular compartment and/or a more intense emission from the cells.

As far as we are aware, no systematic attempt has been made to characterize the glycosaminoglycans in a comprehensive material of chondrosarcomatous tumors. However, the occurrence of sulfated glycosaminoglycans has been examined in a few separate cases. Two chondrosarcomata and one chordoma were studied by *Meyer et al.* (1956). One chondrosarcoma yielded chondroitin-4-sulfate as the only glycosaminoglycan, whereas the other two tumors contained only chondroitin-6-sulfate. *Adams* (1963) isolated chondroitin-4-sulfate from one chondrosarcoma and demonstrated the absence of keratan sulfate. Previous investigations have thus repeatedly shown that chondroitin sulfates occur in chondrosarcomatous tissue, but have failed to disclose the presence of keratan sulfate. It nevertheless seems reasonable to expect also the latter glycosaminoglycan to occur at least in some cases of chondrosarcoma, since it too is an important constituent of normal human cartilage. This assumption is supported by the results of the fractionation study in case 1.

In the latter study the tumor samples showed much the same pattern of radioactivity. This indicates that the malignant cells produced a similar spectrum of sulfated glycoaminoglycans regardless of their location in the tumor. Caution is required, however, when interpreting the significance of the individual fractions, since a different material from ours was used by *Antonopoulos et al.* (1964) when they originally elaborated their microseparation technique. Yet, the presence of well-defined fractions 1 (keratan sulfate) and 3 (chondroitin sulfate), both in the sample of joint cartilage and in the different tumor samples, would seem to indicate that a satisfactory separation of the classical glycosaminoglycans was indeed obtained. If the applicability of the separation method to the present material is accepted on this basis, it follows that keratan sulfate was a constituent of the ground substance of the tumor in case 1. Moreover, the synthesis of this polysaccharide was greatly accelerated in relation to that of chondroitin sulfate(s). An increased content of keratan sulfate in cartilage has previously been found to be characteristic of the ageing process, and has also been reported to occur in pathological processes, *e.g.*, in Marfan's syndrome (*Meyer et al.* 1958). The exact meaning of the occurrence of marked S^{35}-activity in fraction 4 cannot as yet be stated, but may tentatively be interpreted as indicating an alteration in the synthesis of chondroitin sulfate(s) by chondrosarcomatous cells.

There is reason to believe that S^{35}-sulfate incorporation in whole tissue systems *in vitro* adequately reflects the corresponding process *in vivo*. In both cases intact and living cells are required. For example, heating or freezing and thawing of tissue slices cause an abolition of S^{35}sulfate incorporation *in vitro* (*Layton et al.* 1950, *Boström & Månsson* 1953). The absence of S^{35}-uptake in necrotic areas of the tumor samples in the present study is another example; nor were necrotic portions of experimental skin found to incorporate the isotope *in vivo* (*Boström et al.* 1954).

Very little is so far known about the possible relationship between the degree of malignancy of various chondrosarcomas and their production and content of various glycosaminoglycans. If it could be shown, however, that in this respect, qualitative and quantitative differences exist between chondromas and chondrosarcomas, or even between chondrosarcomatous tumors of different degree of malignancy, *in vitro* investigations, along the lines applied or suggested in the present study, might be helpful in establishing the diagnosis and in selecting therapy in individual cases. That the sensitivity of chondrosarcomas to treatment with S^{35}-sulfate *in vivo* may vary widely, is evident from both the literature and our experience (*Boström et al.*, unpublished observations).

SUMMARY

The *in vitro* incorporation of S^{35}-sulfate was studied in samples from two surgically removed chondrosarcomas of long bones; samples of adjacent joint cartilage served as controls.

The utilization of the isotope was determined by biochemical analyses, made visible by autoradiography, and correlated to the morphology of the individual samples.

Incorporation, by the tumor samples, of S^{35}-sulfate into the total glycosaminoglycan fraction exceeded that of the controls by 7 to 15 times in case 1, and by 1.5 to 9 times in case 2.

Determination of the radioactivity of individual glycosaminoglycan fractions in case 1 showed that a similar spectrum of sulfated glycosaminoglycans was produced in all the tumor samples. Keratan sulfate and chondroitin sulfate(s) were the main constituents. The synthesis of keratan sulfate was greatly accelerated in relation to that of chondroitin sulfate(s).

The autoradiograms showed that the uptake of S^{35}-sulfate was due to a pronounced cellular incorporation, both in chondrosarcomatous tissues and in normal cartilage. Variations in cellular content of the tissues was a major factor in causing the differences, in the uptake of the isotope, between the tumor samples and the control samples and also between individual tumor samples.

The significance is discussed of *in vitro* experiments, as a supplement to therapeutic trials with S^{35}-sulfate, in cases of chondrosarcoma.

RESUME

L'incorporation in vitro de sulfate S^{35} a été étudiés sur des prélévements de deux chondrosarcomes d'os long, obtenus chirurgicalement. Des spémens de cartilage articulaire adjacent ont servi de contrôle.

L'utilisation de l'isotope a été déterminée par analyse biochimique rendue visible par autoradiographie et en corrélation avec la morphologie des prélévements individuels.

L'incorporation dans les prélévements de la tumeur de sulfate S^{35} a été effectuée jusque'à ce que la fraction de glycosaminoglycan dépasse celle des spécimens de contrôle de 7 à 15 fois dans le cas 1 et de 1,5 à 9 fois dans le cas 2.

La détermination de la radioactivité des fractions glycosaminoglycan individuelles dans le cas 1 montre qu'un spectre similaire de glycosaminoglycan sulfaté s'est produit dans tous les prélévements de la

tumeur. Des sulfates de kératine ont été les principaux constituants. La synthèse du sulfate de kératine était fortement accélérés par rapport à celle du sulfate de chondroitine.

Les autoradiographies montrent que l'absorption de sulfate S^{35} est due à une incorporation cellulaire prononcée, tant dans les tissus chondrosarcomateux que dans le cartilage normal. Des variations dans le contenu cellulaire des tissue a été un facteur majeur comme cause des variations dans l'absorption de l'isotope entre les différents prélévements de la tumeur.

Il est discuté de l'importance des expériences *in-vitro* comme complément aux essais thérapeutiques avec le sulfate S^{35} dans le cas de chondrosarcome.

ZUSAMMENFASSUNG

Die in vitro Einverleibung von S^{35}-Sulfat wurde an Proben von zwei chirurgisch entfernten Chondrosarkomen langer Rörenknochen studiert. Proben von angrenzendem Gelenksknorpel dienten als Kontrollen.

Die Ausnützung der Isotopen wurde mittels biochemischer Analysen bestimmt, wurde mittels Autoradiographie sichtbar gemacht und in Beziehung zur Morphologie der einzelnen Proben gebracht.

Aufnahme in die Tumorproben von S^{35}-Sulfat in die gesamte Glycosaminoglycan-Fraktion überstieg die der Kontrollen 7 bis 15 mal im 1. Falle, und 1,5 bis 9 mal im 2. Falle.

Bestimmung der Radioaktivitet von einzelnen Glycosaminoglycan-Fraktionen in Fall 1 zeigte, dass ein gleichartiges Spektrum von Sulfat Glycosaminoglycanen in allen Tumorproben erzeugt wurde. Keratansulfat und Chondroitinsulfat(e) waren die Hauptbestandteile. Die Synthese von Keratansulfat war im Verhältnis zu der des Chondroitinsulfat(e) sehr beschleunigt.

Die Autoradiogramme zeigten, dass die Aufnahme von S^{35}-Sulfat einer ausgesprochenen zellulären Einverleibung zuzusprechen war, sowohl in chondrosarkomatösem Gewebe als auch in normalem Knorpel. Unterschiede im Zellgehalt der Gewebe waren ein grösserer Faktor bei der Verursachung der Verschiedenheiten, in der Aufnahme der Isotopen, zwischen den Tumorproben und den Kontrollproben und auch zwischen den einzelnen Tumorproben.

Die Bedeutung der *in vitro* Versuche als eine Ergänzung zu den Behandlungsversuchen mit S^{35}-Sulfat in Fällen von Chondrosarkom wird besprochen.

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