THE ROLE OF FLUORIDE IONS IN GLYCOSAMINOGLYCANS SULPHATION IN CULTURED FIBROBLASTS

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SUMMARY: The purpose of this study was to evaluate sulphur incorporation (by $^{35}$S sulphate) into glycosaminoglycans (GAG) cultures of isolated cells, pericellular substance and medium, and into glycosaminoglycans present in sulphate fibroblasts with NaF added to the culture. In the study, primary cultures of fibroblasts were used, isolated by tissue trypsinization from mice livers. Fibroblasts were cultured with the addition of NaF ([$\text{F}^{-}$] = 0.116 $\times 10^{-3}$ M/dm$^3$) and the addition of NaF and $[^{35}$S]-Na$_2$SO$_4$ (activity $^{35}$S = 30 $\mu$Ci/cm$^3$). Simultaneously with the experimental cultures, control cultures were also examined. The effect of $\text{F}^{-}$ ions on culture growth, protein content in fibroblasts, and their morphometric characteristics were evaluated. Three fractions were isolated from fibroblast cultures: cell, pericellular substance, and medium. From these fractions glycosaminoglycans were isolated. GAG obtained from fibroblasts were electrophoretically separated, resulting in heparan sulphate (HS), dermatan sulphate (DS), and chondroitin sulphates (CS). Even in low concentrations $\text{F}^{-}$ ions have a toxic effect on fibroblast cultures. Growth inhibition and decrease in size, accompanied by a change in shape, were observed. Under the same conditions fluoride ions significantly modified incorporation of $^{35}$S into fibroblasts and GAG from individual fractions of experimental cultures. Analysis of sulphated GAG content in the fibroblasts showed interference by $\text{F}^{-}$ ions, both in their synthesis and metabolism, as well as their diffusion. The results suggest a significant increase in synthesis intensity and/or the degree of DS sulphation and a decrease in the intensity of the process in relation to CS and HS.

Key Words: Cultured fibroblasts; Fluoride ions; Glycosaminoglycans; Sulphation.

INTRODUCTION

Interest in the effect of fluoride compounds on the human body is, to a great extent, due to their use in osteoporosis treatment$^{1-3}$ and prevention of dental caries$^4$. However, research should largely be focused on the effect of fluorides on connective tissue, where fluoride compounds cause changes in collagen$^5-7$ and the metabolism of glycosaminoglycans (GAG)$^5,6,8$, the main basic substance of connective tissue.

The aim of this study was the evaluation of the effects of fluoride ions on sulphur incorporation, in the form of SO$_4^{2-}$ ions, into GAG in fibroblast cultures. The quantity of $\text{F}^{-}$ ions did not exceed the value of the CaF$_2$ and MgF$_2$ solubility product. Qualitative and quantitative reciprocal relations among individual GAGs were examined. Fibroblast cultures were used in the study to evaluate $\text{F}^{-}$ ion effects on culture growth, protein content in cells, and chosen morphometric parameters of fibroblasts.

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MATERIALS AND METHODS

The study was performed using primary fibroblast cultures, isolated by tissue trypsinization\(^9\) from the livers of 60 day old mice of both sexes of Balb c strain. Fibroblasts were cultured on Eagle's medium MEM 1959\(^9\) with fetal calf serum containing antibiotics (penicillin 100 U and streptomycin 20 \(\mu\)g/cm\(^3\)) in an atmosphere of 5\% CO\(_2\) and a temperature of 310\(^\circ\)K. All isolation and fibroblast culture procedures were performed under aseptic conditions in a laminar chamber.

Cultures for evaluation of F\(^-\) ions effect on fibroblasts were carried out by addition of NaF in quantity 0.116 \(\times\) 10\(^{-3}\) M/dm\(^3\); and cultures for evaluation of GAGs sulphation except NaF (in concentration as above) by addition of \([^{35}\text{S}]\)-Na\(_2\)SO\(_4\) with the activity \(^{35}\text{S}\) 30 \(\mu\) Ci/cm\(^3\). Along with the examined cultures, control cultures were performed without NaF, with and without \([^{35}\text{S}]\)-Na\(_2\)SO\(_4\).

Culture growth was determined on days 2, 4, 7, 11 and 14 by counting cells in Bürker's chamber (with prior staining with trypane blue). For microscopic examination and morphometric analysis, the cells were trypsinated from the bottom of the culture vessel. Morphometric evaluation was performed with the VIDS computerized image analysis system (AMS, England).

The Lowry procedure was used to determine protein content in fibroblasts.\(^1\)\(^0\),\(^1\)\(^1\) Fibroblasts for protein content determination were obtained without the use of trypsin.

Cultures to be examined provided 3 fractions (fibroblast, pericellular, and medium).\(^1\)\(^2\) From each fraction GAGs were isolated by the method of Svajcar and Van Robertson \(^1\)\(^3\) with Wosicki's modification.\(^1\)\(^4\) Fibroblast cells were homogenized by use of a pressure press. Then the pericellular and medium cell homogenates were submitted to digestion by papain and afterward deproteinized by TCA solution. Finally, an ethanol solution of potassium acetate was used to precipitate GAG. GAGs obtained from fibroblasts were electrophoretically separated by Wessler's method,\(^1\)\(^5\) consisting of cellulose acetate strips in barium acetate solution (in a Beckman apparatus and a current of 1 mA/cm\(^2\) charge density) and after staining with althian blue and elution with potassium acetate solution. Separated GAG identification was performed on the basis of DS, HS, C4S and C6S patterns electrophoresis (Sigma prod.). In this way, heparan (HS) and dermatan sulphate (DS) were obtained along with the mixture of chondroitin sulphates (CS), namely, chondroitin-4-sulphate (C-4-S) and chondroitin-6-sulphate (C-6-S).

Radioactivity measurements of all samples containing \(^{35}\text{S}\), including also eluates from separated glycosaminoglycans, were performed with a Beckman scintillation counter after placing them on scintillation blotting-paper discs of the same type. Counting efficiency was 70-80\%.

RESULTS

Due to the presence of calcium and magnesium ions in the culture medium, a low concentration of fluoride ions was used in the study, not exceeding the value of CaF\(_2\) and MgF\(_2\) solubility product. This means that the results obtained allow the assessment of fluoride ions effect in an invariable concentration of calcium and magnesium ions in fibroblast cultures, which was of great
significance in eliminating effects of a series of enzymes, particularly those dependent on Mg$^{2+}$ ions presence.

Intensified fibroblasts growth inhibition caused by F$^-$ ions was found during the culture period. Growth characteristics of the fibroblast cultures are shown in Figure 1 as growth curves, allowing determination of the logarithmic growth phase.

Figure 1. Growth curves $\log N = f(t)$ of fibroblast cultures with NaF and control cultures
The data in Table 1 indicate that culture growth index - \( \frac{N}{N_0} \) (\( N \) = the number of cells per 1 cm\(^3\) of the medium at the end of experiment, \( N_0 \) = the number of cells per 1 cm\(^3\) at the beginning of the experiment) decreased in time (for 2-14 days of culturing) in the range 82% to 44% of the values of corresponding control cultures. As a result, in relation to duration, the value of the culture growth rate \( \left( \frac{\ln N/N_0}{T} \right) \) decreased from 74 to 54% with the increase in time value of cell doubling period \( (T_d = \frac{0.6932}{\mu}) \) from 145 to 185%.

### Table 1. The effect of NaF on growth of fibroblast cultures

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Control</th>
<th>Fibroblasts cultured with NaF</th>
<th>Compared with control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( N/N_0 ) \pm S</td>
<td>( \bar{N}/\bar{N}_0 ) \pm S</td>
<td>( \bar{\mu} )</td>
</tr>
<tr>
<td>2</td>
<td>1.50-2.90</td>
<td>2.27 0.61</td>
<td>0.39 49</td>
</tr>
<tr>
<td>4</td>
<td>2.90-6.63</td>
<td>4.58 0.64</td>
<td>0.37 47</td>
</tr>
<tr>
<td>7</td>
<td>4.90-8.70</td>
<td>6.77 0.65</td>
<td>0.27 63</td>
</tr>
<tr>
<td>11</td>
<td>4.63-8.37</td>
<td>6.52 0.62</td>
<td>0.17 101</td>
</tr>
<tr>
<td>14</td>
<td>4.40-8.03</td>
<td>6.29 0.57</td>
<td>0.13 130</td>
</tr>
</tbody>
</table>

* average of 9 measurements † defined in the text

At the same time, in the presence of F\(^-\) ions, alterations in the size and shape of cultured fibroblasts were observed, as shown in Tables 2 and 3. Fibroblast size decreased with the culture's duration, both in the experimental and control cultures, though fibroblasts cultured with NaF were from the onset (after 24 hours) smaller than those in control cultures (Table 2). Relatively greater changes concerned the fibroblasts area and spherical volume \( V = \frac{2}{3} \cdot \text{area} \cdot \text{spherical diameter} \). In comparison with the control culture, the value of these two parameters decreased approximately 20 to 30%. The values of other parameters concerning the size of the fibroblasts, namely, their circumference and spherical diameter \( (D = 2 \cdot \sqrt{\frac{\text{Area}}{\pi}}) \) decreased only 10 to 20%. Alterations in fibroblasts shape parameters (Table 3), namely, their longest dimension and the greatest width seem to be correlated. This dependence is expressed as follows: maximal reduction of the longest dimension corresponds to minimal reduction of the greatest width and vice versa.

The effect of F\(^-\) ions on incorporation and \(^{35}\)S content was studied in 3 systems: in fibroblasts, pericellular substance, and medium (3 culture fractions = system I, in GAGs isolated from these fractions - system II, in GAGs electrophoretically separated from sulphate fibroblasts = system III).

The results in the form of dpm (disintegrations per minute) were calculated per 1 mg of fibroblast cellular protein (protein assay average in corresponding fibroblast culture 2, 4 and 7 day old were considered) and also by percentages corresponding to individual components of the systems examined in relation to

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### TABLE 2. Size parameters of fibroblasts

<table>
<thead>
<tr>
<th>Time (days)*</th>
<th>Control</th>
<th>Fibroblasts cultured with NaF</th>
<th>Compared with control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P ±S</td>
<td>O ±S</td>
<td>D ±S</td>
</tr>
<tr>
<td>2</td>
<td>127.50</td>
<td>51.91</td>
<td>1145 659</td>
</tr>
<tr>
<td>4</td>
<td>113.85</td>
<td>23.95</td>
<td>928 304</td>
</tr>
<tr>
<td>7</td>
<td>103.86</td>
<td>18.32</td>
<td>805 205</td>
</tr>
</tbody>
</table>

*Average of 9 measurements  
P = area (µm²)  
O = circumference (µm)  
D = spherical diameter (µm)  
V = spherical volume (µm³)

### TABLE 3. Shape parameters of fibroblasts

<table>
<thead>
<tr>
<th>Time (days)*</th>
<th>Control</th>
<th>Fibroblasts cultured with NaF</th>
<th>Compared with control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L ±S</td>
<td>B ±S</td>
<td>F ±S</td>
</tr>
<tr>
<td>2</td>
<td>17.06</td>
<td>4.40</td>
<td>0.69 0.14</td>
</tr>
<tr>
<td>4</td>
<td>16.47</td>
<td>2.32</td>
<td>0.74 0.11</td>
</tr>
<tr>
<td>7</td>
<td>15.44</td>
<td>1.51</td>
<td>0.69 0.13</td>
</tr>
</tbody>
</table>

*Average of 9 measurements  
L = longest dimension (µm)  
B = greatest width (µm)  
F = shape factor
total dpm value of a particular system set equal to 100%. The changes of $^{35}$S percentages are shown in Tables 4 to 6.

| Table 4. System I - Percentage of radioactive $^{35}$S in fibroblast cultures |
|----------------------------------|------------------|
| Control                         | With NaF         |
| Fibroblasts                      | 0.4              | 0.4              |
| Pericellulars                    | 3.8              | 2.7              |
| Medium fractions                 | 95.8             | 96.9             |

| Table 5. System II - % of $^{35}$S in the GAG isolated from fibroblast cultures |
|----------------------------------|------------------|
| Control                         | With NaF         |
| Fibroblasts                      | 9                | 17.0 (twice control) |
| Pericellulars                    | 21               | 11.5 (half control)  |
| Medium fractions                 | 71               | 71.5               |

| Table 6. System III - % of $^{35}$S in the GAG isolated from fibroblast cultures |
|----------------------------------|------------------|
| Control                         | With NaF         |
| Heparan Sulphate                 | 40               | 37 (8% < control)  |
| Dermatan Sulphate                | 26               | 42 (81% > control) |
| Chondroitin Sulphate             | 34               | 21 (38% < control) |

The changes in system I seen in Table 4, caused by F$^-$ ions acting on fibroblast cultures, are limited to pericellular substance. A slight decrease in $^{35}$S content is seen in pericellular substance in relation to the control, expressed in a decrease of pericellular substance $^{35}$S percentage from 3.8 to 2.7%. This change is explained in the light of further results, concerned directly with sulphated GAG. These results, obtained in system II, related to sulphated GAG movement among individual fractions of fibroblast cultures are shown in Table 5. In cultures with NaF, a nearly twofold increase (from 9 to 17%) of $^{35}$S content in GAG isolated from pericellular substance was found. This is an obvious sign of F$^-$ ions partly inhibiting sulphated GAG permeation from fibroblasts to pericellular substance. The observed phenomenon may also be the cause of the earlier noted decrease of $^{35}$S content in fibroblast pericellular substance in system I (Table 4). The results concerning content alterations of sulphated GAG contained in fibroblasts in system III are shown in Table 6. As a result in GAG isolated from cells cultured with F$^-$, a 61% increase of $^{35}$S content bound with DS and a 38.8% decrease, respectively, of $^{35}$S content bound with CS and HS was found.

**DISCUSSION**

Cell cultures are a primary research model replacing experimental animals in many areas of biological investigation. In studies concerning the effect of fluoride ions on animal organisms, cultures of different connective tissue cells are most often used - osteoblasts, odontoblasts, and fibroblasts. In the present study fibroblast control cultures grew rapidly with 3 growth phases distinctly seen: the introductory phase (up to 24 hours), the logarithmic phase (from 2nd to 7th day), and stationary phase (from 7th day). The occurrence of a relatively long 5-day logarithmic phase during the growth of fibroblasts culture proved its usefulness in the studies performed here.
The observed inhibition of fibroblasts, intensifying with time, was due to the presence of \( F^- \) ions. Growth inhibition has its onset in the introductory phase and intensifies in the logarithmic phase, which consequently causes a significant decrease of fibroblasts in the stationary phase. This inhibition might indicate that the presence of \( F^- \) ions is favourable for lethal effects, occurring in each growth phase of the culture. A growth inhibition and lethal effects as the result of the action of \( F^- \) ions on fibroblast cultures were previously discussed by Sato et al.,27 Oguro et al.,30 and Veron et al.31

Interesting results were obtained from the morphometric analysis of fibroblasts. Due to fluoride ions, fibroblasts decreased in area and spherical volume in relation to fibroblasts of control cultures. Simultaneously, smaller (in comparison with area and volume alterations) changes of fibroblasts shape (circumference and diameter) that were found might suggest folding of their surface (area). Similarly, Sato et al.27 observed folding of the surfaces of fibroblasts cultured with \( F^- \) ions.

Among numerous research papers on the effects of \( F^- \) ions on cell cultures, there are few reports5,8,32-34 about the impact of these ions on sulphur metabolism and GAG sulphate. It is known that the contents of these latter compounds and the degree of their polymerization and sulphation change as the organism grows older; for instance in the skin, there is an increase of DS content and a decrease in HA content. An increase of DS content in dental tissues and in osseous tissue under the effect of \( F^- \) ions was observed by Susheela and Sharma.32,33

At the same time, these authors found a decrease in the molecular weight of GAG and more pronounced changes caused by \( F^- \) ions in relation to the age of experimental animals. Waddington et al.34 indicate \( F^- \) ions act on odontoblasts in vitro with a tendency to decrease GAG sulfate synthesis. Depending on the \( F^- \) ions concentration used in culture media, 3 or 6 mM/dm³, there was an increase in CS or DS activity, respectively, as for the sulphate GAG content in cells. Simultaneously, they observed a decrease in the molecular weight of proteoglycans isolated from the odontoblasts. Investigating the segments of pig's skin, Ammitzboll et al.5 found no changes in sulphate GAG content in tissue, and only not totally unequivocal changes of CS, DS and HS content.

The study we performed using sulphur in fibroblast cultures introduced into a culture as \( SO_4^{2-} \) ions, focused on individual culture fractions (system I), in GAG isolated from these fractions (system II), and in some sulphated GAG isolated from fibroblasts (system III). These experimental systems are different in the possibilities and type of sulphate ions acceptors in them. In this way, the simplest is the system III in which all components belonging to sulphate GAG (DS, HS and CS), are potential acceptors of \( SO_4^{2-} \) ions. As for system II, apart from these acceptors, there may be, belonging to GAG, but not undergoing sulphation, hyaluronic acid - HA. In system I there is a possibility of occurrence other than GAG, of sulphate ions acceptors and their use in other processes, i.e. after reduction of these ions, that cannot be ruled out.
Changes in system I, as the result of F⁻ ions activity on cultured fibroblasts, are in fact limited only to pericellular substance fraction. The observed decrease in relation to control of ³⁵S content in pericellular substance, due mainly to sulphate GAG movement, without any increase in sulphur content of the fibroblasts, might suggest partial inhibition of GAG permeation from fibroblasts to pericellular substance.

The results in system II illustrated in Table 4 confirm this tendency. Changes concerning a decrease in ³⁵S content in sulphated GAG isolated from pericellular substance and a corresponding increase of its content in tested GAG isolated from fibroblasts, definitely indicate partial inhibition of sulphate GAG permeation from fibroblasts to pericellular substance caused by fluoride ions. The fact that in system I there was no corresponding balance of these changes may be due to the lack of technical potential, combined with an unsatisfactory quantity of stained ³⁵S content in the fibroblast fractions.

The results concerning GAG isolated from individual fractions of the fibroblast cultures were enlarged by qualitative and quantitative analysis of sulfated GAG present in them. The results obtained in system III, illustrated in Table 6, indicate fluoride ion interference, probably not only in the diffusion process of individual sulphated GAG synthetized in fibroblasts in pericellular substance, but also in synthesis and metabolism of the GAG. Changes in the range of ³⁵S content, incorporated into individual sulphate GAG of fibroblasts, caused by F⁻ ions, indicate a significant rise in the intensity of synthesis and/or the degree of DS sulphation and a decrease in the intensity of these processes in relation to CS and HS.

The results of this study concerning the effect of trace amounts of F⁻ ions on fibroblast cultures, indicate an influence on both the growth of these cultures and the size and shape of fibroblasts, as well as synthesis and/or sulphation of sulphated GAG and their diffusion from fibroblasts into pericellular substance.

CONCLUSIONS

1. It appears that even at low concentrations F⁻ ions may have a toxic effect on fibroblast cultures, as seen to their growth inhibition and a decrease in the size of fibroblasts, also accompanied by alterations in their shape.

2. F⁻ ions significantly modify sulphur incorporation, in the form of SO₄²⁻ ions, into glycosaminoglycans isolated from individual fractions of these cultures and into dermatan, heparan and chondroitin sulphates present in fibroblasts.

3. In relation to the above, F⁻ ions cause some changes in the biosynthesis and/or sulphation of glycosaminoglycans in fibroblasts, particularly in relation to an increase in the dermatan sulphate content in relation to heparan and chondroitin sulphates.

4. The observed changes in the intensity of biosynthesis and/or the degree of glycosaminoglycans sulphation in fibroblasts may be accompanied by partial limitation of GAG diffusion outside the cell to pericellular substance.
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