Characterization of Tumor Necrosis Factor Alpha-Induced Alteration of Glycosaminoglycans in Cultured Cells: Comparison among Vascular Smooth-Muscle Cells, Vascular Endothelial Cells, Chang Liver Cells and LLC-PK1 Cells

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We investigated the alteration of glycosaminoglycans (GAGs) induced by recombinant human tumor necrosis factor alpha (rhTNFα) using confluent cultures of bovine aortic smooth-muscle cells, bovine aortic endothelial cells, Chang liver cells and porcine kidney LLC-PK1 cells. It was found that the incorporation of both [^{35}S]sulfate and [^{3}H]glucosamine into GAGs in the trypsinate fraction of the cell layer was significantly decreased by rhTNFα in vascular smooth-muscle cells and vascular endothelial cells; the incorporation of [^{35}S]sulfate was increased but that of [^{3}H]glucosamine was unchanged in Chang liver cells; the incorporation of both [^{35}S]sulfate and [^{3}H]glucosamine was increased by rhTNFα in LLC-PK1 cells. In the conditioned medium, the incorporation of both [^{35}S]sulfate and [^{3}H]glucosamine was not greatly changed by rhTNFα in all tested cell types. Characterization of GAGs revealed that each cell type uniquely altered its GAGs after rhTNFα treatment; the cytokine-induced alteration of each GAG component was not necessarily the same among different cell types. It was therefore concluded that rhTNFα-induced alteration of GAGs is dependent upon cell type.

Keywords cytokine; endothelial cell; glycosaminoglycan; tumor necrosis factor alpha; vascular; vascular smooth-muscle cell

Glycosaminoglycans (GAGs) have been implicated in the physiology and pathology of tissues. For example, in vascular smooth-muscle cells, heparan sulfate inhibits their proliferation[1] and dermatan sulfate activates heparin cofactor II[2] which is a physiological inhibitor of thrombin. The cells accumulate GAGs in the intima lesions of early and late atherosclerosis.[3]

We previously reported that recombinant human tumor necrosis factor alpha (rhTNFα) induces qualitative changes of GAGs in subconfluent cultures of vascular smooth-muscle cells.[4] This suggested that the cytokine derived from macrophages may be involved in vascular lesions such as atherosclerosis.

However, it was not clear whether or not the rhTNFα-induced qualitative changes of GAGs occurs specifically in vascular smooth-muscle cells. In addition, it is possible that rhTNFα-induced alteration may be dependent on components of GAGs, but independent of cell type.

In the present study, we compared first the rhTNFα-induced alteration of GAGs in vascular smooth-muscle cells, confluent *versus* subconfluent. Since it was found that the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into GAGs was changed by rhTNFα in a different way in these two conditions, we next considered that the effect of rhTNFα on GAGs must be characterized by using different cell types. The alteration of GAGs induced by rhTNFα in confluent cultures of bovine aortic smooth-muscle cells, bovine aortic endothelial cells, human Chang liver cells and porcine kidney LLC-PK1 cells was also investigated.

Materials and Methods

Materials Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Nissui Pharmaceutical (Tokyo, Japan) and Cell Culture Laboratories (Cleveland, OH, U.S.A.), respectively. Tissue culture plates and dishes were from Costar (Cambridge, MA, U.S.A.). Bovine serum albumin fraction V (BSA) was obtained from Miles (Kankakee, IL, U.S.A.). Vascular smooth-muscle cells and vascular endothelial cells, derived from bovine aorta, were gifts from Drs. Katuo Sueishi and Yutaka Nakashima (First Department of Pathology, Faculty of Medicine, Kyushu University, Fukuoka, Japan). Chang liver cells and LLC-PK1 cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). rhTNFα (2×10^7 U/mg) was from Genzyme (Cambridge, MA, U.S.A.). D-[1,6-^{3}H(N)]Glucosamine hydrochloride (2282.9 GBq/mmol) and [^{35}S]Na_2SO_4 (carrier-free) were obtained from New England Nuclear (Boston, MA, U.S.A.). Chondroitin sulfate A, hyaluronidase from *Streptomyces hyalurolyticus*, chondroitinase AC and ABC were from Seikagaku Kogyo (Tokyo, Japan). Trypsin (1:250) and pronase were from Difco Laboratories (Detroit, MI, U.S.A.) and Boehringer-Mannheim (Germany), respectively. Nitrous acid and cetylpyridinium chloride (CPC) were purchased from Nacalai Tesque (Kyoto, Japan).

Incorporation of [^{35}S]Sulfate and [^{3}H]Glucosamine into GAGs Vascular smooth-muscle cells, vascular endothelial cells, Chang liver cells and LLC-PK1 cells were each cultured in DMEM supplemented with 10% fetal bovine serum in 24-well culture plates at 37 °C in 5% CO_2 in air until confluent. The medium was discarded and the cell layer was washed twice with DMEM supplemented with 1% BSA. The cell layer was then incubated at 37 °C for 24 h with or without rhTNFα (0.1, 1 or 10 ng/ml) in the presence of both [^{35}S]sulfate (370 kBq/ml) and [^{3}H]glucosamine (100 kBq/ml) in 0.25 ml of DMEM supplemented with 1% BSA. After incubation, the medium was harvested and the cell layer was washed with 0.25 ml of Ca, Mg-free phosphate-buffered saline (CMF-PBS); the wash was then combined with the medium. The cell layer was incubated at 37 °C for 5 min with 0.25 ml CMF-PBS containing 0.25% trypsin and 0.02% EDTA. The trypsinized cell suspension was harvested and the well was washed with 0.25 ml CMF-PBS. The wash was then combined with the cell suspension and centrifuged at $1500 \times g$ for 5 min to obtain the supernatant (trypsinate fraction). The trypsinate fraction includes GAGs derived from the cell surface and from the solubilized extracellular matrix.[5] The trypsinate fraction and the medium were used to determine the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into GAGs by the method of Wasteson *et al.*[6] as follows: the trypsinate and medium were incubated with 3 mg/ml pronase at 50 °C for 3 h. The pronase digest was

mixed with 4 mg/ml carrier chondroitin sulfate A and 0.5% CPC. After incubation at 37 °C for 30 min, the mixture was centrifuged at 1500 × *g* for 10 min to obtain the precipitated GAGs–CPC complexes. The precipitate was dissolved in 0.1 ml 4 M NaCl and re-precipitated by addition of 1.4 ml 80% aqueous ethanol. The precipitate was collected by centrifugation at 1500 × *g* for 10 min and dissolved in 0.4 ml distilled water. The incorporated radioactivities were measured by liquid scintillation counting.

Characterization of GAGs Confluent cultures of vascular smooth-muscle cells, vascular endothelial cells, Chang liver cells and LLC-PK1 cells were treated with or without rhTNFα (3 or 10 ng/ml) for 24 h in 2 ml of DMEM supplemented with 1% BSA in 60 mm dishes in the presence of both [^{35}S]sulfate (740 kBq/ml) and [^{3}H]glucosamine (200 kBq/ml). In another experiment, subconfluent cultures of vascular smooth-muscle cells were treated with rhTNFα (10 ng/ml) in a similar way. After treatment, the medium was collected and the cell layer was washed with 2 ml CMF-PBS; the wash was then combined with the medium. The cell layer was then incubated at 37 °C for 30 min with 2 ml 1% trypsin solution. The cell suspension was harvested and the dish was washed with 2 ml CMF-PBS; the wash was combined with the cell suspension. The trypsinate fraction was obtained by centrifugation at 1500 × *g* for 5 min and an aliquot of either the trypsinate fraction or the collected medium was used for enzymatic or chemical treatments as follows: the aliquot was evaporated to dryness and subjected to pronase digestion according to Yamauchi *et al.*[7] Digestion was performed in 0.4 M sodium acetate buffer (pH 5.0) containing 0.01 M $CaCl_2$ for 72 h at 37 °C. Fresh enzyme at 1 mg/ml was added to the reaction mixture three times at 24-h intervals. At the end of the incubation, the digest was boiled for 5 min to inactivate pronase. The pronase digest was re-evaporated to dryness and treated with hyaluronidase at 50 turbidity-reducing units at 60 °C for 6 h in 0.4 ml 20 mM acetate buffer (pH 5.0); chondroitinase AC or ABC at 0.4 U/ml at 37 °C for 2 h in 0.5 ml 20 mM Tris–HCl buffer (pH 8.0)[8]; or 2.5% nitrous acid in 16.5% acetic acid in a total volume of 0.4 ml at 30 °C for 2 h.[9] The radiolabeled GAGs resistant to each treatment were isolated by CPC precipitation; the radioactivity in GAGs sensitive to each treatment was calculated by subtracting the radioactivity (dpm) of GAGs resistant to the treatment from that (dpm) of untreated GAGs. The radioactivity incorporated into GAGs sensitive to hyaluronidase was considered to represent hyaluronate; that sensitive to chondroitinase AC was considered to represent chondroitin sulfates (A plus C); that sensitive to chondroitinase ABC, but resistant to chondroitinase AC, was considered to represent dermatan sulfate; and that sensitive to nitrous acid was considered to represent heparan sulfate.

Statistical Analysis Results were analyzed for statistical significance by Student's *t*-test. *p* values of less than 0.05 were considered to indicate statistically significant differences.

Results

Table I shows the effect of rhTNFα on the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into GAGs of subconfluent and confluent cultures of vascular smooth-muscle cells (the data for confluent cultures were calculated from those in Table II). Different responses to rhTNFα were observed for the subconfluent and the confluent cultures. For example, the incorporation of [^{35}S]sulfate into chondroitin sulfates in the trypsinate fraction of the cell layer was significantly increased by rhTNFα in the subconfluent cells but significantly decreased in the confluent cells; the incorporation of [^{3}H]glucosamine into the GAG was unchanged in the subconfluent cells but markedly decreased in the confluent cells. In the medium, rhTNFα induced no significant alteration of the incorporation of both [^{35}S]sulfate and [^{3}H]glucosamine into all components of GAGs in the confluent cells, while the [^{35}S]sulfate and [^{3}H]glucosamine incorporations were altered by rhTNFα in the subconfluent cells.

Since it was shown that rhTNFα treatment results in different changes in GAGs between confluent and subconfluent smooth-muscle cells, the effect of rhTNFα on the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into GAGs of four different cell types, vascular smooth-muscle cells, vascular endothelial cells, Chang liver cells and LLC-PK1 cells, was compared using confluent cultures. As shown in Fig. 1, the rhTNFα-induced alteration of GAGs was dependent on the cell type. In the trypsinate fraction of the cell layer, rhTNFα slightly but significantly decreased the incorporation of both [^{35}S]sulfate and [^{3}H]glucosamine into GAGs in vascular smooth-muscle cells and vascular endothelial cells; however, the cytokine significantly increased the [^{35}S]sulfate incorporation in both Chang liver cells and LLC-PK1 cells and the [^{3}H]glucosamine incorporation in the latter cell type. In the medium, rhTNFα did not induce marked changes in the incorporation of both [^{35}S]sulfate and [^{3}H]glucosamine.

Table II shows the effect of rhTNFα on the incorporation of [^{35}S]sulfate and [^{3}H]glucosamine into every component of GAGs in vascular smooth-muscle cells. In the trypsinate

TABLE I. Alteration of the Incorporation of [^{35}S]Sulfate and [^{3}H]Glucosamine into Glycosaminoglycans by rhTNFα in Subconfluent and Confluent Cultures of Vascular Smooth-Muscle Cells

Fraction	Trypsinate		Medium	
	Subconfluent	Confluent	Subconfluent	Confluent
Total				
^{35}S	1.167±0.043[b]	0.825±0.016[c]	1.214±0.052[a]	1.000±0.018
^{3}H	0.851±0.027[b]	0.633±0.008[c]	1.036±0.050	0.930±0.013
Hyaluronate				
^{3}H	—	—	0.541±0.073[c]	1.120±0.336
Chondroitin sulfates (A plus C)				
^{35}S	1.386±0.067[c]	0.855±0.029[a]	4.696±0.397[c]	1.036±0.039
^{3}H	1.008±0.043	0.604±0.015[c]	1.571±0.110[c]	1.013±0.029
Heparan sulfate				
^{35}S	0.786±0.046[b]	0.864±0.039[a]	1.518±0.111[a]	0.954±0.027
^{3}H	0.666±0.036[c]	0.696±0.022[c]	1.062±0.133	0.827±0.078
Dermatan sulfate				
^{35}S	0.966±0.047	0.817±0.049[a]	0.095±0.079[b]	—
^{3}H	0.291±0.075[c]	0.592±0.053[c]	0.212±0.063[c]	0.827±0.078

Subconfluent and confluent cultures of vascular smooth-muscle cells were incubated at 37 °C for 24 h with or without rhTNFα at 10 ng/ml in the presence of both [^{35}S]sulfate and [^{3}H]glucosamine. Values are means±S.E. of 5 samples expressed as the ratio of the radioactivity (dpm) in the rhTNFα treatment to that (dpm) in the corresponding control. Significantly different from the corresponding control, *a*) $p<0.05$; *b*) $p<0.01$; *c*) $p<0.001$. —, impossible to calculate.

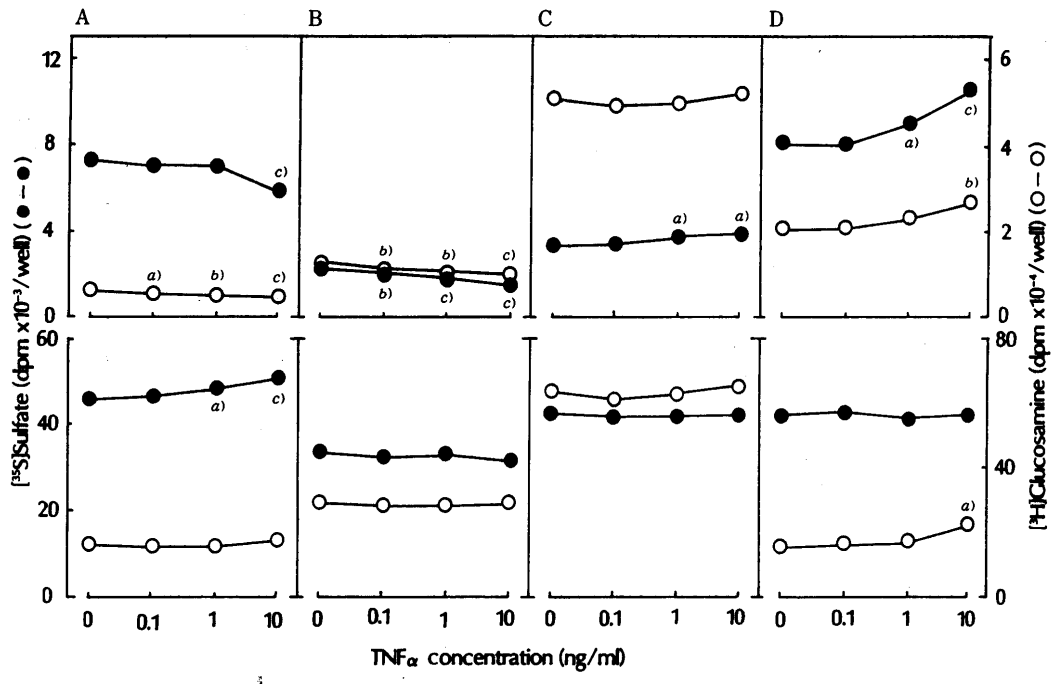

Fig. 1. Effect of rhTNFα on the Incorporation of [^{35}S]Sulfate (●) and [^{3}H]Glucosamine (○) into GAGs in Either the Trypsinate Fraction of the Cell Layer (Upper Panels) or the Medium (Lower Panel) of Vascular Smooth-Muscle Cells (A), Vascular Endothelial cells (B), Chang Liver Cells (C) and LLC-PK1 Cells (D)

Confluent cultures of each cell type were incubated at 37 °C for 24 h with or without TNFα (0.1, 1.0 or 10 ng/ml) in the presence of both [^{35}S]sulfate and [^{3}H]glucosamine. Values are means ± S.E. of 4 samples. Significantly different from the corresponding control, *a*) $p<0.05$; *b*) $p<0.01$; *c*) $p<0.001$.

TABLE II. Characterization of rhTNFα-Induced Alteration of the Incorporation of [^{35}S]Sulfate and [^{3}H]Glucosamine into Glycosaminoglycans of Confluent Cultures of Vascular Smooth-Muscle Cells

Fraction	Trypsinate		Medium	
	Control (dpm/well $\times 10^{-2}$)	rhTNFα (dpm/well $\times 10^{-2}$)	Control (dpm/well $\times 10^{-3}$)	rhTNFα (dpm/well $\times 10^{-3}$)
Total				
^{35}S	557 ±4	459 ± 8[b]	832 ±13	833 ±24
^{3}H	625 ±6	396 ± 2[b]	202 ± 5	188 ± 7
^{35}S/^{3}H	0.892	1.161	4.113	4.425
Hyaluronate				
^{3}H	N.D.	N.D.	14 ± 5	16 ± 7
Chondroitin sulfates (A plus C)				
^{35}S	300 ±2	257 ±10[a]	657 ±22	679 ±23
^{3}H	283 ±8	170 ± 4[b]	129 ± 5	131 ± 7
^{35}S/^{3}H	1.063	1.508	5.096	5.196
Heparan sulfate				
^{35}S	183 ±3	158 ± 6[a]	603 ±12	575 ±22
^{3}H	246 ±8	171 ± 5[b]	27 ± 4	26 ± 2
^{35}S/^{3}H	0.746	0.926	22.720	22.489
Dermatan sulfate				
^{35}S	87 ±4	69 ± 2[a]	N.D.	N.D.
^{3}H	76 ±4	45 ± 3[b]	31 ± 3	25 ± 1
^{35}S/^{3}H	1.135	1.556		

Confluent cultures of vascular smooth-muscle cells were incubated at 37 °C for 24 h with or without rhTNFα at 10 ng/ml in the presence of both [^{35}S]sulfate and [^{3}H]glucosamine. Values are means ± S.E. of 5 samples. Significantly different from the corresponding control, *a*) $p<0.05$; *b*) $p<0.001$. N.D., not detected.

fraction of the cell layer, rhTNFα significantly decreased the incorporation of both [^{35}S]sulfate and [^{3}H]glucosamine into chondroitin sulfates, heparan sulfate and dermatan sulfate; hyaluronate was not detected; the ratio of [^{35}S]sulfate to [^{3}H]glucosamine, a marker of the sulfation of GAGs, was increased by rhTNFα in all sulfated GAGs. In the medium, no significant change was induced by rhTNFα.

The effect of rhTNFα in vascular endothelial cells is shown in Table III. In the trypsinate fraction of the cell layer, heparan sulfate was the major component in this cell type. The incorporation of [^{35}S]sulfate into both chondroitin sulfates and heparan sulfate was significantly decreased by rhTNFα; that of [^{3}H]glucosamine was increased in chondroitin sulfates but decreased in heparan sulfate; the ratio of [^{35}S]sulfate to [^{3}H]glucosamine was decreased in both chondroitin sulfate and heparan sulfate. Hyaluronate and dermatan sulfate were not detected. In

TABLE III. Characterization of rhTNFα-Induced Alteration of the Incorporation of [^{35}S]Sulfate and [^{3}H]Glucosamine into Glycosaminoglycans of Confluent Cultures of Vascular Endothelial Cells

Fraction	Trypsinate		Medium	
	Control (dpm/well $\times 10^{-2}$)	rhTNFα (dpm/well $\times 10^{-2}$)	Control (dpm/well $\times 10^{-3}$)	rhTNFα (dpm/well $\times 10^{-3}$)
Total				
^{35}S	203 ± 2	142 ± 4[b]	869 ± 8	829 ± 8[a]
^{3}H	1281 ±12	1186 ±29[a]	502 ±13	468 ±14
^{35}S/^{3}H	0.159	0.119	1.731	1.771
Hyaluronate				
^{3}H	N.D.	N.D.	45 ± 6	62 ± 6
Chondroitin sulfates (A plus C)				
^{35}S	41 ± 3	31 ± 2[a]	727 ±11	647 ±19[a]
^{3}H	172 ±14	332 ±15[b]	276 ± 7	249 ±12
^{35}S/^{3}H	0.236	0.097	2.269	2.603
Heparan sulfate				
^{35}S	161 ± 2	110 ± 4[b]	719 ± 8	674 ±22
^{3}H	1002 ±13	926 ± 3[a]	228 ± 5	251 ± 9
^{35}S/^{3}H	0.160	0.119	3.151	2.683
Dermatan sulfate				
^{35}S	N.D.	N.D.	N.D.	N.D.
^{3}H	N.D.	N.D.	24 ± 2	11 ± 5[a]
^{35}S/^{3}H	—	—	—	—

Confluent cultures of vascular endothelial cells were incubated at 37 °C for 24 h with or without rhTNFα at 3 ng/ml in the presence of both [^{35}S]sulfate and [^{3}H]-glucosamine. Values are means ± S.E. of 5 samples. Significantly different from the corresponding control, *a*) $p<0.05$; *b*) $p<0.001$. N.D., not detected.

TABLE IV. Characterization of rhTNFα-Induced Alteration of the Incorporation of [^{35}S]Sulfate and [^{3}H]Glucosamine into Glycosaminoglycans of Confluent Cultures of Chang Liver Cells

Fraction	Trypsinate		Medium	
	Control (dpm/well $\times 10^{-2}$)	rhTNFα (dpm/well $\times 10^{-2}$)	Control (dpm/well $\times 10^{-3}$)	rhTNFα (dpm/well $\times 10^{-3}$)
Total				
^{35}S	353 ± 3	389 ± 2[c]	777 ±21	854 ±14[a]
^{3}H	6635 ±107	6972 ±127	1048 ±10	1042 ±31
^{35}S/^{3}H	0.053	0.056	0.741	0.819
Hyaluronate				
^{3}H	N.D.	N.D.	329 ±10	279 ±20
Chondroitin sulfates (A plus C)				
^{35}S	113 ± 3	141 ± 5[c]	640 ±15	704 ±11[b]
^{3}H	2223 ± 92	2506 ± 75[a]	736 ±11	726 ±27
^{35}S/^{3}H	0.051	0.056	0.868	0.970
Heparan sulfate				
^{35}S	162 ± 1	175 ± 3	666 ±20	736 ±13[a]
^{3}H	2766 ± 78	2919 ± 71	528 ± 7	465 ±31
^{35}S/^{3}H	0.058	0.060	1.261	1.583
Dermatan sulfate				
^{35}S	547 ± 3	573 ± 2	N.D.	N.D.
^{3}H	729 ± 62	648 ± 21	31 ± 8	42 ±10
^{35}S/^{3}H	0.075	0.088	—	—

Confluent cultures of Chang liver cells were incubated at 37 °C for 24 h with or without rhTNFα at 10 ng/ml in the presence of both [^{35}S]sulfate and [^{3}H]glucosamine. Values are means ± S.E. of 5 samples. Significantly different from the corresponding control, *a*) $p<0.05$; *b*) $p<0.01$; *c*) $p<0.001$. N.D., not detected.

the medium, the incorporation of [^{35}S]sulfate into chondroitin sulfates was significantly decreased by rhTNFα.

Table IV shows the alteration of GAGs induced by rhTNFα in Chang liver cells. rhTNFα significantly increased the incorporation of the [^{35}S]sulfate and [^{3}H]glucosamine into chondroitin sulfate in the trypsinate fraction of the cell layer; the ratio of [^{35}S]sulfate to [^{3}H]-glucosamine was unchanged. In the medium, rhTNFα significantly increased [^{35}S]sulfate incorporation into both chondroitin sulfates and heparan sulfate.

The effect of rhTNFα in LLC-PK1 cells is shown in Table V. In the trypsinate fraction of the cell layer, rhTNFα significantly increased the incorporation of both [^{35}S]-sulfate and [^{3}H]glucosamine into chondroitin sulfates and heparan sulfate; the ratio of [^{35}S]sulfate to [^{3}H]-glucosamine was unchanged. In the medium, rhTNFα significantly increased the [^{3}H]glucosamine incorporation into chondroitin sulfates and heparan sulfate.

Discussion

The importance of changes in the amount and types of GAGs has been shown to be involved in the pathology of

TABLE V. Characterization of rhTNFα-Induced Alteration of the Incorporation of [^{35}S]Sulfate and [^{3}H]Glucosamine into Glycosaminoglycans of Confluent Cultures of LLC-PK1 Cells

Fraction	Trypsinate		Medium	
	Control (dpm/well $\times 10^{-2}$)	rhTNFα (dpm/well $\times 10^{-2}$)	Control (dpm/well $\times 10^{-3}$)	rhTNFα (dpm/well $\times 10^{-3}$)
Total				
^{35}S	716 ± 8	879 ± 16[b]	610 ± 14	643 ± 11
^{3}H	3734 ± 48	4777 ± 72[b]	231 ± 7	340 ± 6[b]
^{35}S/^{3}H	0.191	1.161	2.722	1.890
Hyaluronate				
^{3}H	N.D.	N.D.	71 ± 7	82 ± 4
Chondroitin sulfates (A plus C)				
^{35}S	134 ± 14	224 ± 10[a]	458 ± 18	491 ± 11
^{3}H	474 ± 47	932 ± 32[b]	91 ± 9	142 ± 7[b]
^{35}S/^{3}H	0.291	0.247	5.291	3.476
Heparan sulfate				
^{35}S	575 ± 9	665 ± 17[a]	561 ± 15	588 ± 11
^{3}H	2807 ± 3	3470 ± 89[a]	172 ± 7	252 ± 4[b]
^{35}S/^{3}H	0.204	0.191	3.349	2.340
Dermatan sulfate				
^{35}S	N.D.	N.D.	N.D.	N.D.
^{3}H	N.D.	N.D.	N.D.	N.D.
^{35}S/^{3}H	—	—	—	—

Confluent cultures of LLC-PK1 cells were incubated at 37 °C for 24 h with or without rhTNFα at 10 ng/ml in the presence of both [^{35}S]sulfate and [^{3}H]glucosamine. Values are means ± S.E. of 5 samples. Significantly different from the corresponding control, *a*) $p<0.01$; *b*) $p<0.001$. N.D., not detected.

several diseases[10-16] and vascular lesions such as atherosclerosis[17,18] and thrombosis.[19] It has also been reported that cytokines regulate vascular smooth-muscle cell GAGs quantitatively and qualitatively. Transforming growth factor beta (TGFβ) stimulates the synthesis of GAGs by arterial smooth-muscle cells.[20] Stimulation of GAG synthesis induced by endothelial cell conditioned medium in vascular smooth-muscle cells is due to TGFβ.[21,22] We recently reported that TNFα qualitatively changes GAGs in subconfluent cultures of vascular smooth-muscle cells.[4] This suggested that macrophages, which are present in atherosclerotic plaques with smooth-muscle cells[23] and produce TNFα, may be involved in the formation of atherosclerotic vessels. In the present study, firstly, the TNFα-induced alteration of GAGs was compared between confluent and subconfluent cultures of vascular smooth-muscle cells, since the response of the cells to TGFβ differs under these conditions.[24] It was found that rhTNFα induced different changes in GAGs in vascular smooth-muscle cells in these two stages. The data suggest that the alteration induced by TNFα, as well as TGFβ, is dependent on the stage at least in this cell type and may partly explain the various changes in GAGs in the atherosclerotic vessels.

It is an important observation that the rhTNFα-induced alteration of GAGs is cell-type-specific and/or GAG component-specific. It was shown that rhTNFα altered the incorporation of [^{35}S]sulfate and/or [^{3}H]glucosamine into GAGs in the trypsinate fraction of the cell layer of all tested cell types including vascular smooth-muscle cells, vascular endothelial cells, Chang liver cells and LLC-PK1 cells; however, no marked change was observed in the medium fraction. The ratio of [^{35}S]sulfate to [^{3}H]glucosamine in the cell layer was increased in vascular smooth-muscle cells, decreased in vascular endothelial cells and unchanged in Chang liver cells and LLC-PK1 cells. These results clearly indicate that rhTNFα has a capacity for altering the production of sugar chains and/or their sulfation and/or the anchorage of the GAGs to the cell layer, depending on the cell type. Although the mechanism by which rhTNFα causes the different changes in GAG metabolism among the four cell types tested is not clear, an assumption can be made that each cell type has an original system for the regulation of GAG metabolism and/or a unique system of response to rhTNFα, which may be partly dependent on the species. However, these remain to be elucidated.

On the other hand, the effect of rhTNFα was not GAG-component specific. The incorporation of [^{35}S]sulfate into chondroitin sulfate of the trypsinate fraction of the cell layer was significantly decreased by rhTNFα in vascular smooth-muscle cells and endothelial cells but significantly increased in Chang liver cells and LLC-PK1 cells; the incorporation of [^{3}H]glucosamine into the GAG was significantly decreased by the cytokine in vascular smooth-muscle cells but significantly increased in vascular endothelial cells, Chang liver cells and LLC-PK1 cells. In heparan sulfate, the incorporation of both [^{35}S]sulfate and [^{3}H]glucosamine was significantly decreased by rhTNFα in vascular smooth-muscle cells and endothelial cells, unchanged in Chang liver cells and significantly increased in LLC-PK1 cells. These results suggest that the response of each GAG to rhTNFα, in terms of metabolism, is not the same among different cell types. In other words, rhTNFα-induced alteration is not necessarily dependent on the GAG component.

The present study has shown that (i) rhTNFα causes changes GAGs which differs between confluent and subconfluent cultures of vascular smooth-muscle cells, (ii) rhTNFα-induced alteration of GAGs is different among the various cell types, and (iii) the metabolism of each GAG component can respond to rhTNFα in different ways in the different cell types. It is thus suggested that the TNFα-

induced alteration of GAGs in vascular smooth-muscle cells,[4] which may be involved in the pathology of blood vessels, is based on the particular relationship between TNFα and the cell type. The physiological and/or pathological significance of these multifunctional effects of TNFα on GAG metabolism should be investigated further.

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