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# Effects of wheat germ agglutinin and concanavalin A on the accumulation of glycosaminoglycans in pericellular matrix of human dermal fibroblasts. A comparison with insulin★✪

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**The effect of insulin, wheat germ agglutinin (WGA), peanut agglutinin (PNA) and concanavalin A (ConA) on [$^3$H]glucosamine incorporation into pericellular glycosaminoglycans (GAGs) was investigated in two lines of cultured human dermal fibroblasts. Insulin and WGA stimulated [$^3$H]glucosamine incorporation into hyaluronic acid (HA) and heparan sulphate (HS) without any alteration of chondroitin sulphate (CS) and dermatan sulphate (DS) contents. ConA increased [$^3$H]glucosamine incorporation into HS, CS and DS, but had no effect on [$^3$H]glucosamine incorporation into HA. PNA affected neither the content, nor the composition of GAGs. In contrast to PNA, ConA and WGA stimulated glycolysis and demonstrated an evident antiproliferative effect on dermal fibroblasts. Thus, both the insulin-like action of WGA and ConA on cultured dermal fibroblasts and the differences between the effects of lectins on modulation of GAGs synthesis appear to be determined by their chemical structure.**

It is well known that some plant lectins are able to demonstrate an evident insulin-mimetic effect on insulin receptors, glucose and amino acid uptake, as well as on lipid and carbohydrate metabolism in various cell types [1], including connective tissue. Besides, it

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**Abbreviations:** ConA, concanavalin A; CS, chondroitin sulphate; DS, dermatan sulphate; GAG, glycosaminoglycan; HA, hyaluronic acid; HS, heparan sulphate; PNA, peanut agglutinin; WGA, wheat germ agglutinin.

has been shown that wheat germ agglutinin (WGA) and concanavalin A (ConA), like insulin itself, induce the synthesis of specific messenger RNA (p33-mRNA) in hepatoma cells [2] and mimic the direct effects of insulin on nuclear envelope phosphorylation [3]. However, WGA and ConA inhibit cell growth of transformed and normal cell lines in the presence of serum, suppress cell attachment, their spreading and migration [4–6], i.e. demonstrate the effects which are not characteristic for insulin.

To extend information on the insulin-like effects of lectins we have compared the effect of insulin and lectins on the content and composition of glycosaminoglycans (GAGs) in two lines of cultured human dermal fibroblasts.

Alterations of cell behavior are always accompanied by changes of the extracellular matrix (ECM), and, in particular, of its glycosaminoglycan (GAG) components: hyaluronic acid and sulphated GAGs (heparan sulphate, chondroitin sulphate, dermatan sulphate and keratan sulphate). The number and type of carbohydrate side chains, their length and pattern of sulphation vary with cell state, differentiation, and in disease [7–9]. GAGs ensure a water-salt balance of ECM, participate in the organization of its structure and provide the reservoir of various growth factors and cytokines, facilitating their interaction with the cell surface high affinity receptors.

It is known that a number of growth factors and hormones, including insulin [10–12] are able to modulate GAGs synthesis and metabolism. However, the influence of insulino-mimetic plant lectins on GAGs synthesis is unclear and the investigations available, did not include the comparative aspects of WGA, ConA and insulin on GAGs [13–16].

In the present paper the effect of insulin was compared with that of ConA ($\alpha$-D-man, $\alpha$-D-glc), WGA (D-glcNAc)2,NeuNAc) and peanut agglutinin (PNA ($\beta$-D-gal(1-3)-D-galNAc) on [$^3$H]glucosamine incorporation into hyaluronic acid (HA), heparan sulphate (HS), chondroitin sulphate (CS) and dermatan sulphate (DS) of pericellular matrix of confluent dermal fibroblasts. Also we examined the effect of lectins on cell growth and lactate production, which was assessed as an indicator of metabolic response.

## MATERIALS AND METHODS

***Materials***. Cell culture medium (EMEM) and necessary additives were purchased from Sigma, with the exception of serum (FCS, mycoplasma-tested), which was obtained from Diagnosticum (Ukraine). Trypsin, soybean trypsin inhibitor, chondroitinase ABC and AC, heparitinase I, orgelase, chondroitin sulphate A, heparan sulphate, cetylpyridinium chloride, D(+)galactose and methyl-$\alpha$-D-manno pyranoside were from Sigma. Lectins were purchased from BioMakor (Israel). Crystalline porcine insulin (Sigma) was purified by gel filtration on Sephadex G-50, fine. Pronase E was from Merck, lactate dehydrogenase and hyaluronic acid-K-salt was purchased from Fluka Chemie AG, and gelatin and DEAE-cellulose (cap. 0.54) from Serva. D-[1-$^3$H]Glucosamine was from Amersham Int. (sp. act. 3.10 Ci/mmole). All other chemicals used were of the highest quality available.

***Cell culture***. Primary cultures obtained from 2 healthy male volunteers (23 and 39 years old) by skin-explant method were grown in the EMEM medium containing 20% FCS, 4 mM glutamine, 50 $\mu$g/ml streptomycin, 50 U/ml penicillin and 1$\mu$g/ml Amphotericin B. Secondary cultures were grown in EMEM with 10% FCS (without antibiotics) and with gelatin as a growth substrate. Cells were routinely subcultured (1:4) in 90 mm dishes every 10 days.

***Experimental procedure.*** Both cell lines were examined twice, i.e. at the 5th and 10th passage; each experiment was carried out with single subcultures (cell count coefficient of variation 6–7%). For the experiments, fibroblasts ($5 \times 10^4$) were plated in 40 mm dishes, and were grown to confluence (6–7

days). Monolayers were washed, and 1.5 ml portions of the following incubation medium were added: 4 mM glutamine, 500 ng/ml bovine serum albumin, 1% FCS, and [$^3$H]glucosamine (3 $\mu$Ci) in EMEM, with or without alternatively: 0.5 $\mu$g insulin, 3 $\mu$g/ml ConA, 3 $\mu$g/ml WGA, and 5 $\mu$g/ml PNA. The cells were incubated in 5% $CO_2$ in air for 48 h at 37$^0$C. The incubation medium was aspirated and used for enzymatic determination of lactate [17].

To prepare the cell-associated (pericellular) GAGs [12], 1 ml of 0.05% trypsin and 0.45% NaCl was added to the washed cell layer and incubated for 15 min. The cells were resuspended and aspirated, then the dish was washed with 1 ml of 0.05% soy bean trypsin inhibitor, 0.1 M Tris/HCl, pH 7.9, the cell suspension was combined with wash liquid and centrifuged (500 g, 2–3 min). Then 1 ml of 0.1 M Tris/HCl, pH 7.9, with 1.5 mg/ml of pronase E, 0.6 mg/ml of hyaluronic acid-K-salt and 0.6 mg/ml of chondroitin sulphate A was added to the supernatant and incubated for 18 h at 55°C. The reaction was stopped by placing the tubes in a boiling water bath for 2–3 min, and the solution was kept frozen at –20°C until required. After defrosting and intensive dialysis against water for 24 h and against 0.01 M Tris/HCl, pH 8.4, for 24 h the solution was applied onto DEAE-cellulose columns (2 ml). Elution was carried out with NaCl gradient (0.10 M, 0.28 M, 0.4 M and 0.7 M) in 0.01 M Tris/HCl, pH 8.4, as described previously [18]. The material which was eluted with 0.10 M NaCl is the residual glycoproteins, it was not shown and was not considered in the work. GAGs were eluted with 0.28 M, 0.4 M and 0.7 M NaCl. Fractions of 3 ml were treated with cetylpyridinium chloride and alcohol. $^3$H-Radioactivity was determined by liquid scintillation counting. [$^3$H]GAGs were desalted and concentrated on Centriprep-3 (Whatman), subjected to gel filtration on Sephadex G-50 and identified by means of specific enzymes and nitrous acid degradation [19]. The following identification criteria were used: (a) GAG sensitive to chondroitinase ABC (EC 4.2.2.4), orgelase (EC 3.2.1.36) and resistant to nitrous acid and heparitinase I (EC 4.2.2.8) was designated HA; (b) GAG sensitive to heparitinase I and nitrous acid, resistant to chondroitinase ABC, chondroitinase AC (EC 4.2.2.5) and orgelase was designated HS; (c) GAG equally sensitive to both chondroitinase ABC and AC, resistant to heparitinase I and nitrous acid, was referred to as CS; (d) material sensitive to chondroitinase ABC, resistant to chondroitinase AC was designated DS. HA appeared in fractions No. 3–6, HS in No. 13–15 and CS/DS in No. 19–20 (Fig. 4). In order to determine [$^3$H]glucosamine incorporation separately into DS and CS, fractions No. 19–20 were pooled and digested by chondroitinase AC. The remaining radioactivity represented $^3$H-DS, and the difference between total radioactivity of fractions No. 19–20 and that in $^3$H-DS was ascribed to $^3$H-CS.

Construction of the dose-dependent curves for insulin and lectins (0.01–30.0 $\mu$g/ml) was based on estimation of [$^3$H]glucosamine incorporation into total pericellular GAGs eluted with 0.7 M NaCl directly after 0.1 M NaCl. The effect of methyl-$\alpha$-D-mannopyranoside (MeMan) and D(+)galactose (Gal) on ConA effect was estimated by measuring of [$^3$H]glucosamine incorporation into total pericellular GAGs.

For the determination of antiproliferative effects of ConA, WGA and PNA the fibroblasts were seeded (5 $\times$ 10$^3$ cells/cm$^2$) in EMEM with 10% FCS, 4 mM glutamine and antibiotics. After 24 h the cells were washed and transferred to EMEM with 5% FCS with or without, alternatively, 3 $\mu$g/ml ConA, 3 $\mu$g/ml WGA or 5 $\mu$g/ml PNA. The final saturation confluent density was determined at that cell concentration at which no further increase in cell number under non-limiting growth conditions was noted in three separate cell counts. Time doubling was determined as $T \times \lg 2/\lg (N/N_0)$, where T is time growth, $N_0$ is cell count per cm$^2$ at the beginning of logarithmic

phase, N is the same in dex at the end of log a rithmic phase.

Since the differences between the passages and between both cell lines in the incorporation of [$^3$H]glucosamine into HA and sulphated GAGs under all used treatments of cells were insignificant: (*P* was equal to 0.51 and 0.49, 0.38 and 0.34, 0.38 and 0.42, 0.44 and 0.46, 0.32 and 0.39 for basal conditions and under insulin, WGA, ConA and PNA treatment, respectively), the data were presented as the means of the re sults of two in dependent ex per i ments in trip li cate for both cell lines together. Statistical analysis was performed using Student's *t*-test.

## RESULTS

As can be seen (Fig. 1) insulin, ConA and WGA increased [$^3$H]glucosamine incorporation into total pericellular GAGs of cultured fibroblasts in a dose-dependent manner. fur ther com par a tive stud ies we used PNA at a con cen tra tion of 5.0$\mu$g/ml, be cause at higher levels of PNA GAGs synthesis was even slightly decreased. The ConA stimulation of [$^3$H]glucosamine incorporation into total GAGs was abol ished by 10 mM MeMan (pos itive con trol), but not by 20 mM Gal (neg a tive control). MeMan or Gal alone were without any effect on [$^3$H]glucosamine incorporation into GAGs (Fig. 2). MeMan supressed also the ConA-induced stimulation of lactate production (not shown).

Figure 3 illustrates the effect of lectins and insulin on lactate production by cultured fibroblasts. Insulin and WGA increased lactate pro duc tion ap prox i mately to the same extent (49.5% and 40.0%, re spec tively), whereas stimulation by ConA amounted only to 20%, and PNA did not in flu ence lac tate pro duc tion at all.

Typical examples of the ion-exchange chroma tog ra phy pro files of pericellular [$^3$H]GAGs of dermal fibroblasts following insulin or

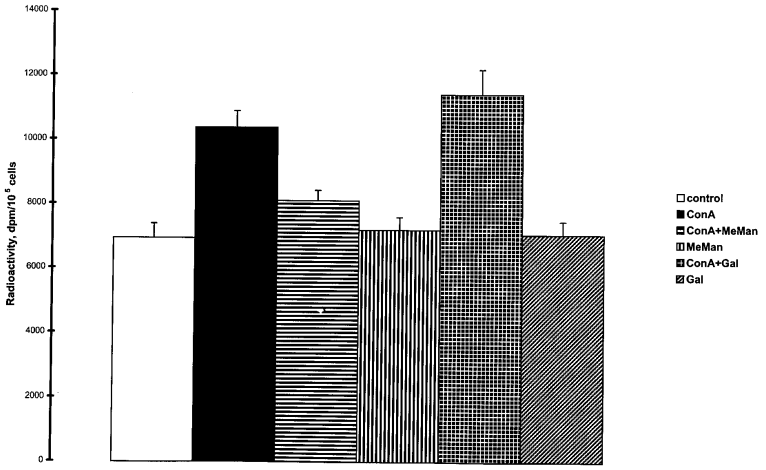


**Fig ure 1. Ef fect of in su lin, ConA, WGA and PNA on [$^3$H]glucosamine in cor po ra tion into to tal pericellular GAGs of der mal fibroblasts.**

Con flu ent monolayers were ex posed to in creas ing con cen tra tions (0.01–30.0$\mu$g/ml) of in su lin and lectins in EMEM sup ple mented with 1% FCS for 48 h. For de tails see "Ma te rials and Methods". Data are ex pressed as means of 2 in dependent ex per i ments (5th and 10th pas sage) in trip li cate for both cell lines. S.E. did not e x ceed 10% of the mean.

Basing on these re sults we used 0.5$\mu$g/ml in su lin and 3.0$\mu$g/ml ConA or WGA for fur ther experiments. PNA did not alter [$^3$H]glucosamine in cor po ra tion into to tal GAGs, and for ConA treatment are presented in Fig. 4. The comparison of [$^3$H]GAGs elution profiles of the cells treated with lectins (data for PNA and WGA are not shown) or insulin and the

untreated cells shows no essential qualitative alterations in GAG components. However, quantitative differences are evident (Fig. 5). [$^3$H]Glucosamine incorporation into HA was increased about two-fold under the influence of insulin and WGA. The increase in [$^3$H]glucosamine incorporation into HS upon insulin, ConA or WGA treatment was by 1.7, 2.8 and 1.8, respectively, but only ConA stimulated [$^3$H]glucosamine incorporation into CS (1.5

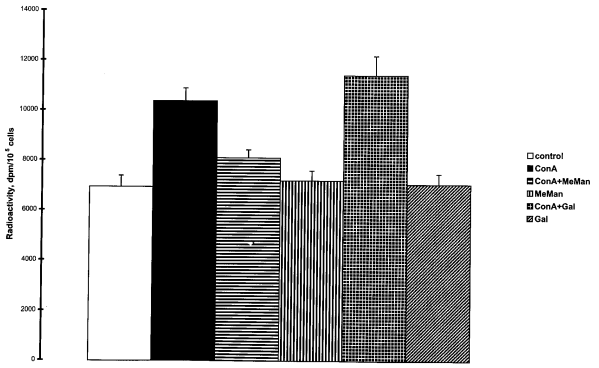


**Figure 2. Effect of methyl-$\alpha$-D-mannopyranoside (MeMan) and D(+)galactose (Gal) on stimulation by ConA of [$^3$H]glucosamine incorporation into total pericellular GAGs.**

Confluent fibroblasts were exposed to 3$\mu$g/ml of ConA in EMEM supplemented with 1% FCS for 48 h in the presence or absence of 10 mM MeMan or 20 mM of Gal. Samples were processed as described in "Materials and Methods". Data are expressed as means ± S.E. of 2 independent experiments (5th and 10th passage) in triplicate. ***significant at $P < 0.01$, as compared to control

fold) and DS (1.6 fold). PNA did not affect incorporation of [$^3$H]glucosamine into any pericellular GAG consistently with the data presented in Fig. 1.

The effect of ConA, WGA and PNA on fibroblast proliferation is demonstrated in Fig. 6. WGA and ConA inhibited the growth of fibroblasts, mainly at the lag-phase of cell growth, and, consistently, the population doubling times were increased. The confluent density measured after 15 days was significantly decreased ($P < 0.02$) under the influence of ConA (23007 ± 1449 cells/cm$^2$, n = 12) as compared to control (31200 ± 2496 cells/ cm$^2$, n = 12), while WGA administration did not reduce final cell density (28211 ± 2229 cells/cm$^2$, n = 12). PNA did not affect proliferation of fibroblasts, and all indices studied were the same as under control conditions.

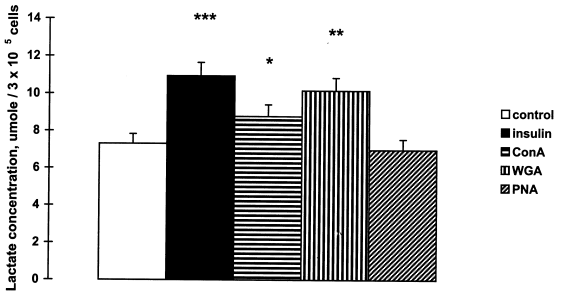


**Figure 3. Effect of insulin, ConA, WGA and PNA on lactate production by dermal fibroblasts.**

Confluent monolayers were exposed to 0.5$\mu$g/ml of insulin, 3$\mu$g/ml of ConA, 3$\mu$g/ml of WGA or 5$\mu$g/ml of PNA in EMEM supplemented with 1% FCS for 48 h. Data presentation as in Fig. 2. ***Significant at $P < 0.01$, **significant at $P < 0.02$, *significant at $P < 0.05$, as compared to control.

## DISCUSSION

According to our data, ConA and WGA stimulate lactate production by confluent fibroblast cultures, and the level of stimulation is comparable to that with insulin, i.e. it is consistent with the opinion on the insulin-like effects of these lectins, formulated earlier [1], and developed by more recent investigations [2, 3, 20].

Insulin, WGA and ConA stimulate pericellular GAGs synthesis, however WGA, like insulin, does not affect [$^3$H]glucosamine incorporation into CS and DS, as shown for chick embryonic fibroblasts [15, 16]. ConA, unlike insulin and WGA, stimulates [$^3$H]glucosamine incorporation only into sulphated GAGs, which is in line with the effect of ConA on chondrocyte cultures [14]. A great (approx. 3 fold) increase of HS synthesis on ConA treatment is consistent with the evident prolongation of population doubling time (approx. 1.7 fold) and reduced confluent density (approx. 1.3 fold) of fibroblasts by ConA.

The link be tween in creased HS-proteoglycans expression and the reduction of proliferative activity in var i ous cell types has been dem on strated in a number of investigations [7–9].

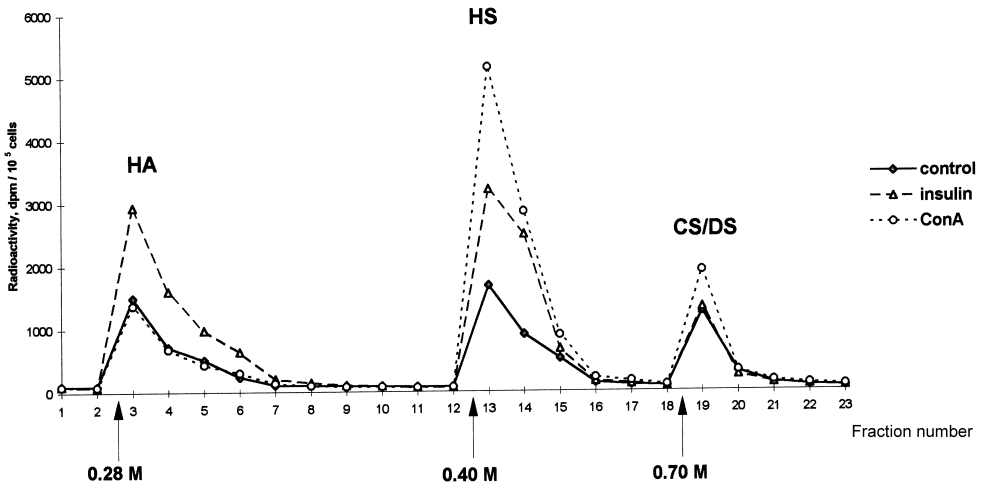


**Fig ure 4. Typ i cal ex am ple of DEAE-cellulose chro ma tog ra phy pro files of pericellular [$^3$H]GAGs of der-mal fibroblasts treated with in su lin and ConA.**

Con flu ent monolayers were ex posed to 0.5 $\mu$g/ml in su lin or 3 $\mu$g/ml ConA in EMEM sup ple mented with 1% FCS for 48 h. GAGs were sep a rated as de scribed un der "Ma te rials and Methods". Ar rows mark NaCl con c entration.

Despite the known di rect ac tion of insulino-mimetic ConA and WGA on in su lin re cep tors [21, 22], the available evidence demonstrates a dif fer ence be tween WGA, ConA and in su lin not only on GAGs synthesis, but on general cell behavior, activity of proteinases, energy me tab o lism and other pro cesses [2–6, 10–16]. Moreover, it was shown that the insulin-like activity of ConA and WGA (stimulation of [$^3$H]aminoisobutyrate uptake) could be elic-ited in the Madin-Darby canine kidney cells even in the ab sence of spe cific in su lin-binding sites [20]. On the other hand, the differences in the ef fects of the two lectins are re peat edly noted. The ConA and WGA treat ment in some tissues resulted in opposite influence on cell growth, as it was shown for nerve fi bers [23], in which ConA stim u lated and WGA in hib ited for ma tion of fi bers per neu ron. Al though high concentrations of either lectin induce apo-ptosis, the mech a nism of their ac tion is dif fer-ent. ConA was shown to break the mitochon-drial membrane potential [24], while WGA binds to the cell surface sialic acid, which is to be es sen tial for its ac tion on cell phys i ol ogy and, in particular, on the regulation of GAGs synthesis.

Moreover, WGA binding to O-GlcNAc-mo-dified proteins may explain the similarity in action of WGA and insulin due to cross-talk between glycosylation and insulin-mediated phosphorylation cas cade [26]. A pro posed im-pli ca tion of O-GlcNAc transferase in the de vel-opment of glucose (or glucosamine)-induced insulin-resistance, in particular, in terms of glycogen synthesis, is especially interesting because UDP-glucosamine is a direct pre cur-sor for GAGs synthesis.

It was shown that mod u la tion of GAGs syn-thesis is mediated *via* phosphatidylinositol 3-kinase [27], which is activated by ConA, WGA and insulin. However, also in this re-spect, the mech a nism of their ac tion is dif fer-ent. ConA acts through the G-protein-coupled and phosphotyrosine-related signalling path-ways, and insulin only *via* the phospho-tyrosine way [28]. The extracellular response to WGA (in terms of the re lease of re ac tive ox-

ygen species) occurs also without involvement of G-proteins in phosphatidylinositol 3-kinase activation [29].

Thus, it is possible that ConA affects GAGs synthesis and their accumulation in different ways, i.e. *via* the activation of phospha-

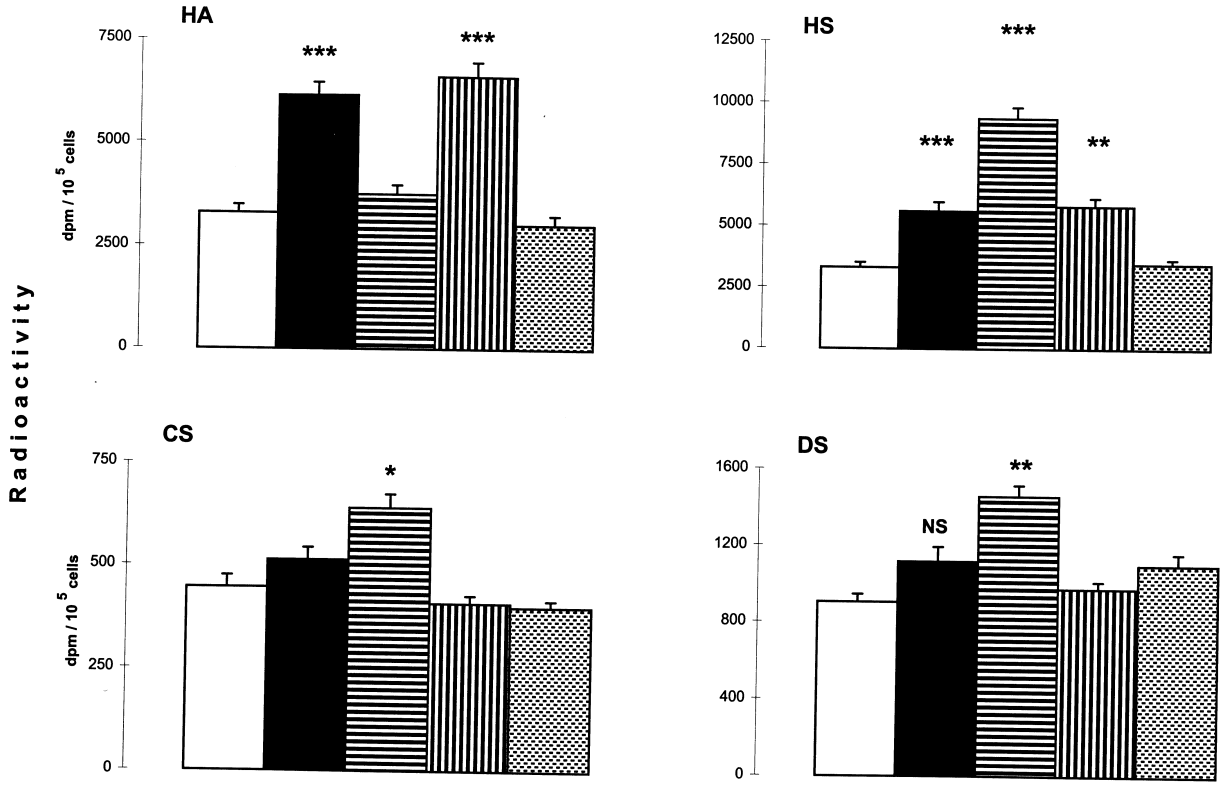


**Figure 5. Effect of insulin (■), ConA (▤), WGA (▥) and PNA (▨) on [$^3$H]glucosamine incorporation into pericellular HA, HS, CS and DS of confluent dermal fibroblasts.**

Separation of GAGs as described under "Materials and Methods". ***Significant at $P < 0.01$, **significant at $P < 0.02$, *significant at $P < 0.05$, NS, nonsignificant, as compared to control.

The synthesis of sulphated GAGs, unlike that of hyaluronic acid, takes place in the Golgi, and then the mature proteoglycans are transported to cell surface. It has been reported that some animal mannose lectins regulate this process [30]. Therefore it seems that ConA influences accumulation of sulphated GAGs by affecting their traffic to pericellular matrix.

Besides, ConA down-regulates the expression of the gene encoding the membrane type-1 matrix metalloproteinase and thereby inhibits the activation of pro-matrix metalloproteinase type 2 [31], which is known to degrade various extracellular matrix proteins, including CS/DS-proteoglycans.

tidylinositol 3-kinase through both G-protein-coupled and phosphotyrosine-related signalling pathways, *via* modulation of proteoglycan transport to the cell membrane and *via* regulation of matrix degradation. WGA, in contrast, modulates GAGs synthesis by activation of phosphatidylinositol 3-kinase only through the phosphotyrosine-related pathway; moreover it acts intracellularly due to its binding to the cytosolic and nuclear O-GlcNAc-modified proteins.

As to PNA, we did not observe any insulin-mimetic stimulation of lactate production and GAGs synthesis. Although PNA has been reported to stimulate proliferation of various cell types [32, 33], but in contrast to the effect

of ConA and WGA, in our experiments we did not observe any alteration of fibroblast growth on PNA treatment.

The results obtained allow to increase the list of the insulin-like effects of WGA and ConA in the field of GAGs synthesis in human

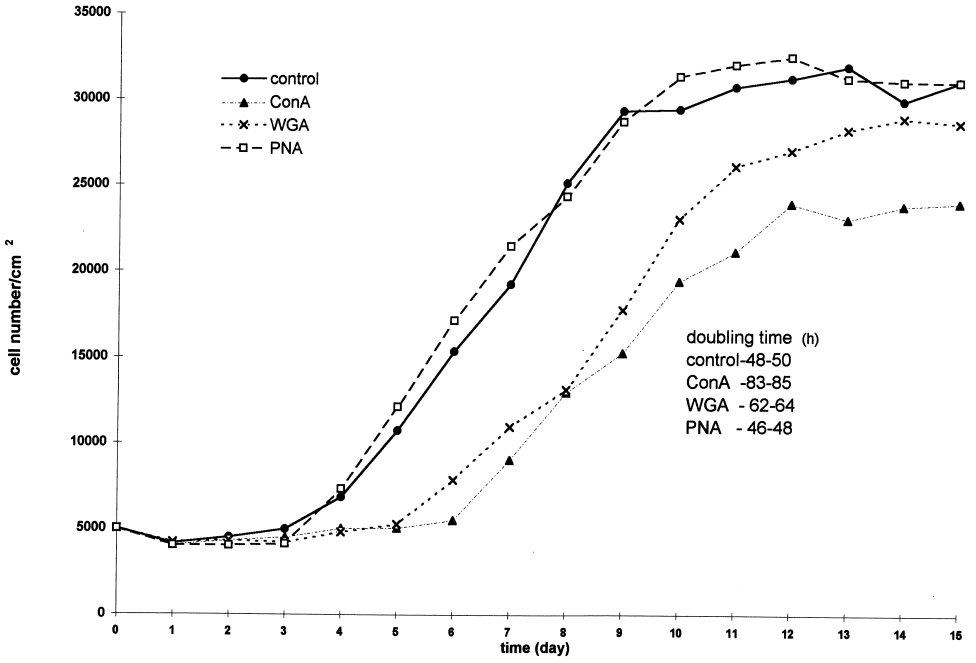


**Figure 6. Antiproliferative effects of ConA, WGA and PNA on dermal fibroblasts.**

Cells were seeded at $5 \times 10^3/cm^2$ in EMEM supplemented with 10% FCS and antibiotics on 40 mm dishes. After 24 h the medium was changed to EMEM with 5% FCS with or without 3 $\mu$g/ml of ConA, 3 $\mu$g/ml of WGA or 5 $\mu$g/ml of PNA. The medium was changed every second day and the cells were counted every day. Data are expressed as the means of two independent experiments (5th and 10th passage) in triplicate together for both cell lines. The S.E. did not exceed 10% of the mean.

Undoubtedly, the effect of ConA and WGA on pericellular GAGs and lack of any response to PNA are determined by differences in their structure. Due to these differences lectins bind to different carbohydrate determinants of cell components, which, in turn, leads to various effects, some of which mimic insulin stimulation of glycosaminoglycan synthesis, while others may be induced independently from lectin action on insulin receptors.

As to the specificity of ConA, a functional mimicry between carbohydrate and peptide ligands of ConA has recently been demonstrated [34]. The peptide ligand revealed interaction properties and energetics equivalent to those of the carbohydrate ligand, and it may provide an additional binding site for ConA.

dermal fibroblasts and simultaneously point to the specific effect of ConA.

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