FEBS Letters 395 (1996) 170–173 FEBS 17635

# In vivo modulated *N*-acyl side chain of *N*-acetylneuraminic acid modulates the cell contact-dependent inhibition of growth

J. Raimund Wieser[a,*], Anja Heisner[a], Peer Stehling[b], Franz Oesch[a], Werner Reutter[b]

[a]*Institut für Toxikologie, Obere Zahlbacherstraße 67, D-55131 Mainz, Germany*
[b]*Institut für Molekularbiologie und Biochemie, Arnimallee 22, D-14195 Berlin-Dahlem, Germany*

Received 8 August 1996

**Abstract Sialylation of plasma membrane glycoproteins is thought to be involved in the regulation of differentiation and in the process of tumorigenesis. Here we show that sialylation also affects cell-cell contact-dependent growth regulation. When cultured in the presence of non-physiological synthetic sialic acid precursors, human diploid fibroblasts no longer exhibited density-dependent inhibition of growth. Concomitantly, increased sialylation of contactinhibin, a glycoprotein involved in density-dependent inhibition of growth, was observed. These results indicate that sialidase-resistant sialic acid modifications lead to dysregulated growth control. The modifications have been induced by *N*-propanoyl and other *N*-acyl derivatives of D-mannosamine.**

*Key words:* Sialic acid; *N*-Propanoyl-D-mannosamine; Density-dependent inhibition of growth; Contactinhibin; In vivo modulation

## 1. Introduction

Differential glycosylation of cell surface compounds represents a powerful system in the regulation of cell recognition and interaction. Typically located at the non-reducing terminus of oligosaccharides, sialic acid plays a crucial role in this control and recognition system. It either acts as a specific recognition site, e.g. as a binding determinant of the ligands for cell adhesion molecules, selectins [1], or exerts a masking effect by blocking underlying structures, e.g. by preventing the breakdown of serum glycoproteins [2,3]. As components of complex carbohydrates, sialic acids occur in a great variety of forms in microorganisms as well as in higher animals, and their occurrence is both species-dependent and tissue-regulated. Variations in sialic acids may have profound biological consequences, e.g. alterations in virus binding to cells [4,5] and in cell adhesion [6], or the determination of developmental processes [7]. We have shown previously that contact-dependent inhibition of the growth of non-transformed human fibroblasts is mediated by the plasma membrane glycoprotein contactinhibin [8] and its receptor [9]. In addition, it has been observed that contactinhibin is synthesized in a highly sialylated form [10]. From earlier studies it is known that terminal β-glycosidically linked galactose residues on *N*-linked oligosaccharide side chains are indispensable for proper growth inhibitory activity [11]. We therefore cultured human diploid fibroblasts in the presence of structurally altered sialic acid precursors, e.g. *N*-propanoyl-D-mannosamine, and investigated whether this treatment affected the contact-dependent inhibition of growth.

We showed that *N*-acyl substitutions at the C-5 position of sialic acid results in the repression of contact-dependent inhibition of growth, most likely due to the inability of human fibroblast sialidases to remove sialic acids from contactinhibin.

## 2. Materials and methods

### 2.1. Cell culture

Human diploid lung fibroblasts were cultured as described earlier [11] with the modification that CG-medium (Vitromex, Vilshofen, Germany) was used at 0.5% FCS (Roth, Karlsruhe, Germany) instead of DMEM at 10% FCS.

### 2.2. Isolation of intracellular and plasma membrane localized contactinhibin

Membrane localized contactinhibin was separated from intracellular contactinhibin by a combination of biotinylation of cell surface sialic acids, immunoprecipitation and avidin affinity separation as described by Wieser et al. [10].

### 2.3. Synthesis of sialic acids derivatives

The sialic acid precursors (*N*-propanoyl-D-mannosamine, *N*-butanoyl-D-mannosamine and *N*-pentanoyl-D-mannosamine) used in this study were synthesized and characterized as described by Keppler et al. [5].

### 2.4. Proliferation assays

These were performed according to Gradl et al. [9]. Briefly, $5 \times 10^3$ cells were seeded per well of a microtiter plate in DMEM/0.5% FCS and cultured for 24 h. Thereafter, immobilized contactinhibin isolated as described, or alternatively, glutaraldehyde-fixed cells [8] were added in DMEM/10% FCS and after a culture period of 24 h cells were then cultured for a further 4 h in the presence of 0.25 μCi [$^3$H]thymidine (20 Ci/mmol, NEN, Germany) and processed to determine the amount of radioactivity incorporated into DNA.

### 2.5. Western blot detection of contactinhibin and of glycoprotein associated sialic acids

Contactinhibin was detected on Western blots with polyclonal *anti*-contactinhibin antibodies [8] and alkaline phosphatase-conjugated *anti*-rabbit antibodies (Sigma, Deisenhofen, Germany). Lectin-based detection of glycoprotein-bound sialic acids (all reagents from Boehringer Mannheim) was performed with digoxigenin-conjugated lectins followed by *anti*-digoxigenin antibodies according to the manufacturer's instructions.

### 2.6. Detection of sialic acids

Sialic acids were determined using a commercially available kit (Boehringer Mannheim) according to the manufacturer's instructions.

### 2.7. Protein quantification

Protein was determined using the bicinchoninic acid assay as described by Smith et al. [12].

## 3. Results and discussion

In previous studies we observed that the addition of contactinhibin to sparsely seeded test cells resulted in pronounced

*Corresponding author. Fax: (49) (6131) 23 05 06.
E-mail: wieser@vzdmzy.zdv.uni-mainz.de


*PII* S0014-5793(96)01029-0

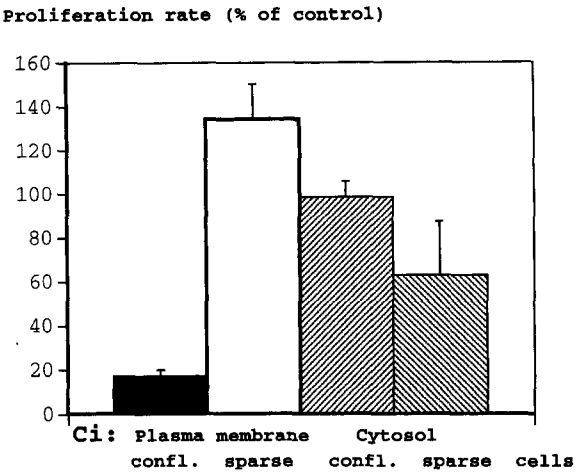


Fig. 1. Proliferation rate of human diploid fibroblasts in the presence of immobilized contactinhibin (Ci) from various sources (i.e. plasma membrane and intracellularly localized contactinhibin from confluent and sparse cells, respectively). $5 \times 10^3$ cells were seeded per microtiter well in the presence of immobilized contactinhibin for 24 h. After a 4 h pulse with [$^3$H]thymidine, cells were processed for measurement of radioactivity incorporated into DNA. Values are the means ± S.E. of 6 independent determinations and are given as percent of controls (cells cultured in the presence of silica beads without contactinhibin).

inhibition of growth [8]. However, for proper growth inhibitory activity, contactinhibin has to be added to the test cells in immobilized form, and most importantly, unmasked, terminal β-glycosidically linked galactose residues have to be present on its *N*-linked oligosaccharides. In addition, we observed that contactinhibin is synthesized in highly sialylated form, and that cell membrane-localized contactinhibin from confluent cells was less sialylated than contactinhibin from sparse cells [10]. We therefore investigated the growth-inhibitory activities of the differently sialylated forms of contactinhibin. Contactinhibin from sparse and confluently grown human fibroblasts was separated into its intracellular and cell membrane-localized forms, immobilized by covalent coupling to derivatized silica beads [13] and added to sparsely seeded human fibroblasts. The proliferation assay revealed that only cell membrane localized contactinhibin from confluently grown cells (i.e. low-sialylated contactinhibin) inhibited growth of the test cells (Fig. 1). Intracellular contactinhibin from either sparse or confluently grown cells (i.e. highly sialylated contactinhibin) was without significant effects, while membrane-localized contactinhibin from sparse cells, which is also highly sialylated, consistently stimulated growth, suggesting the existence of membrane-localized species of contactinhibin in sparse cells which differ structurally from highly sialylated intracellular forms.

These results show that highly sialylated contactinhibin is not involved in growth inhibition; they also indicate the importance of activation (i.e. unmasking of the β-glycosidically linked galactose residues) of contactinhibin by desialylation. Previous studies have revealed the existence of a novel plasma membrane-localized sialidase with substrate specificity for both glycoproteins and gangliosides (unpublished results). This enzyme is suggested to be involved in the conversion of plasma membrane-localized contactinhibin from its highly sialylated, biologically inactive to its low-sialylated, growth-inhibitory form, which occurs when sparsely cells grow to confluence. From experiments performed by other groups it is known that modifications of the *N*-substituent of sialic acids results in reduced cleavage of the glycosides or in their complete resistance to the action of sialidases [14–16]. Since neuraminic acid is taken up to only a small extent by cultured cells, the modulation of sialic acids in vivo requires the administration of the respective hexosamine precursors, which have been shown to be taken up and incorporated efficiently as sialic acid analogues into glycoproteins [5,14].

As a next step we therefore investigated the effects of the chemically synthesized, unphysiological sialic acid precursor, *N*-acyl-D-mannosamine, on density-dependent cell growth regulation. Fig. 2 shows that, depending on the duration of pretreatment with *N*-pentanoyl-D-mannosamine, a marked increase in the relative proliferation rate at increasing cell density was observed, indicating a loss of density-dependent inhibition of growth. This effect was most pronounced in cells pretreated with the precursor for more than 7 days, while no preincubation, or preincubation for shorter periods, was without effect. Similar effects have been observed with other *N*-acyl-D-mannosamine derivatives, i.e. *N*-propanoyl- and *N*-butanoyl-neuraminic acid, while the physiological *N*-acetyl derivative was without effect (data not shown). The observed requirement for a long preincubation period to achieve substantial effects is in agreement with the finding of an extremely low turnover rate of contactinhibin [10].

Contactinhibin was found in earlier studies to be the only cell membrane glycoprotein which inhibits growth in a contact-dependent fashion [8]. Since contactinhibin exerts its growth-inhibitory action exclusively via its *N*-glycans, which are not affected by glutardialdehyde fixation, addition of glu-

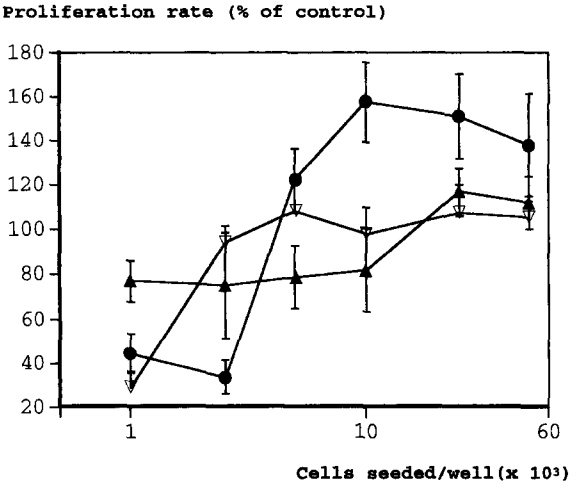


Fig. 2. Influence of *N*-pentyl-D-mannosamine on the proliferation rate of human diploid fibroblasts seeded at different cell densities. Human fibroblasts were seeded at the cell densities indicated on the *x*-axis, and cultured for 24 h. After a 4 h pulse with [$^3$H]thymidine, cells were processed for measurement of radioactivity incorporated into DNA. ▽: cells seeded in the presence of *N*-pentanoyl-D-mannosamine (0.1 mM) without pretreatment; ▲: cells seeded in the presence of *N*-pentanoyl-D-mannosamine (0.1 mM) pretreated for 5 days with 0.1 mM *N*-pentanoyl-D-mannosamine; ●: cells seeded in the presence of *N*-pentanoyl-D-mannosamine (0.1 mM) pretreated for 18 days with 0.1 mM *N*-pentanoyl-D-mannosamine. Values are the means ± S.E. of 6 independent determinations and are given as percent of controls (cells seeded without 0.1 mM *N*-pentanoyl-D-mannosamine).

tardialdehyde-fixed cells to test cells represents a simple means to imitate high cell density in sparse cell cultures and concomitantly, to study contactinhibin-mediated inhibition of growth [11]. We therefore cultured cells in the presence of *N*-acyl-D-mannosamine derivatives, then fixed and tested them for growth inhibitory activity. As shown in Fig. 3, cells preincubated with the synthetic derivatives (the results obtained with *N*-propanoyl-D-mannosamine as precursor are shown as an example) had only one-fifth of the growth-inhibitory activity of the control cells. In order to test the possibility that contactinhibin might exhibit altered expression or altered sialylation in pretreated cells, Western blots were performed with *anti*-contactinhibin antibodies and with the lectins specific for α-2,3- and α-2,6-linked sialic acids, MAA [17] and SNA [18], respectively. Fig. 4 (α-Ci) shows that the pretreatment with the hexosamine analogues does not alter the expression of contactinhibin at the protein level. However, there was no reactivity of contactinhibin with the lectin SNA (Fig. 4, SNA), while reactivity with MAA was reduced (Fig. 4, MAA; no glycoproteins other than contactinhibin react in this gel region with the described lectins: unpublished results), indicating that the sialic acids are significantly modified and are no longer recognized by the lectins. On the other hand, determination of total sialic acids revealed increased amounts of sialic acids on contactinhibin from pretreated cells (Fig. 4, SIA). These results suggest that, in agreement with findings reported by other authors on prokaryotic sialidases, the modified sialic acids are resistant to human fibroblast glycoprotein sialidases. Since desialylation of contactinhibin is a crucial step in the establishment and maintenance of growth control, simple, sialidase-resistant modifications of sialic acid residues may result in impaired growth regulation. In agreement with this, variations of sialic acids were observed in some malignant tumors [19]. In addition, these results provide evidence that the sialic acid-specific lectins used in this study display the narrowest specificity observed so far, in that they not only discriminate between different linkages, but their binding is also greatly affected by simple modifications of the sialic acid moiety.

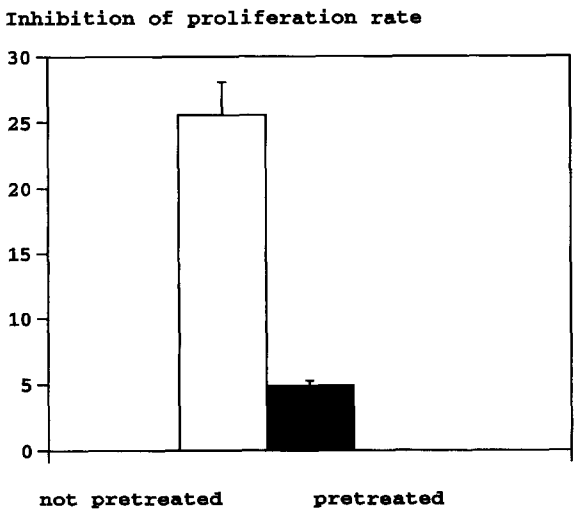


Fig. 3. Proliferation rate of human diploid fibroblasts in the presence of glutardialdehyde-fixed human fibroblasts. $5 \times 10^3$ cells were seeded per microtiter well in the presence of glutardialdehyde-fixed human fibroblasts for 24 h. After a 4 h pulse with [$^3$H]thymidine, cells were processed for measurement of radioactivity incorporated into DNA. Not pretreated: cells to be fixed were grown confluently in the absence of *N*-propanoyl-D-mannosamine. Pretreated: cells to be fixed were grown confluently in the presence of *N*-propanoyl-D-mannosamine (0.1 mM) for 7 days. Values are the means ± S.E. of 4 independent determinations and are given as fold inhibition of proliferation rate. Comparable results were obtained with the other hexosamine analogues.

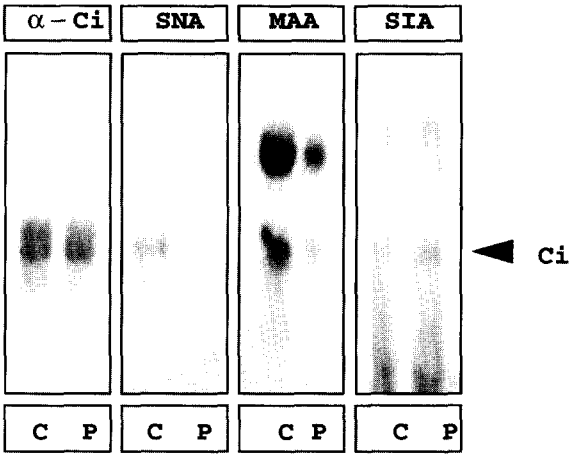


Fig. 4. Analysis of the expression of contactinhibin and of contactinhibin-associated sialic acids in human fibroblasts cultured for 7 days in the absence (C) or presence (P) of *N*-propanoyl-D-mannosamine (0.1 mM). α-Ci: Western blot detection of contactinhibin with anti-contactinhibin-antibodies. SNA: Western blot detection of α-2,6-linked sialic acids with the lectin from *Sambucus nigra*. MAA: Western blot detection of α-2,3-linked sialic acids with the lectin from *Maackia amurensis*. SIA: Western blot detection of total sialic acids. Ci: Contactinhibin. Comparable results were obtained with the other hexosamine analogues.

In summary, the results obtained in this study show for the first time that sialic acids are indirectly involved in contact-dependent inhibition of growth and that in vivo-modulation of sialic acid by new *N*-acyl derivatives of D-mannosamine results in a loss of density-dependent growth control.

*Acknowledgements:* This study was supported by a grant from the Deutsche Forschungsgemeinschaft and the Bundesministerium für Bildung und Forschung and is part of the Ph.D. thesis of H.A.

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