Altered glycosaminoglycan production in cultured osteogenesis-imperfecta skin fibroblasts

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(Received 9 February 1983/Accepted 25 March 1983)

Collagen and glycosaminoglycan syntheses were studied in skin fibroblasts cultured from patients with osteogenesis imperfecta (OI) and from age-matched controls. Collagen synthesis (measured as protein-bound [^3H]hydroxyproline) was decreased in all four OI cell lines studied in the present experiments, comprising 16–24% of total protein synthesis (40% in normal cells). Hyaluronic acid production in OI skin fibroblasts per cell was higher than in age-matched controls, but the production of sulphated glycosaminoglycans was at the normal level. Thus the ratio of the hyaluronic acid and sulphated-glycosaminoglycan radioactivities was markedly higher in OI cultures than in control cultures, especially at the exponential phase of growth where the synthesis of hyaluronic acid was highest. Hyaluronic acid in OI had a normal molecular weight when determined by gel filtration on Sepharose 2B. The removal of high-molecular-weight hyaluronic acid from the medium by hyaluronidase had no effect on the rate of collagen secretion in OI cell line 1 (A.T.C.C. 1262), in which the rate of collagen secretion was lowest.

We have shown (Turakainen *et al.*, 1980) that more hyaluronic acid is produced, and hyaluronate synthetase activity is higher, in skin fibroblasts cultured from osteogenesis-imperfecta (OI) patients than in fibroblasts from control persons. Similar findings had been previously reported for two other heritable connective-tissue disorders, the Marfan (Matalon & Dorfman, 1968) and Hurler (Germinario *et al.*, 1973) syndromes. Our previous studies were carried out by using confluent cultures, and the synthesis of hyaluronic acid was compared with protein synthesis by using simultaneous labelling of glycosaminoglycans with [^3H]glucosamine and proteins with [^{14}C]glycine. Later, we and others (Steinmann *et al.*, 1979; Barsh & Byers, 1981; Barsh *et al.*, 1982) noticed decreased collagen synthesis in several osteogenesis-imperfecta cell lines. The high relative synthesis of hyaluronic acid could be due to decreased protein synthesis. Furthermore, culture conditions (especially cellular density) markedly affect the synthesis of connective-tissue polysaccharides, high cell density favouring decreased synthesis (Hopwood & Dorfman, 1979; Ninomiya & Nagai, 1979; Hronowski & Anastasiades, 1980). Estimation of cell density by visual inspection, which was used previously, is difficult and can lead to

considerable errors. This is true especially if the patient cultures are compared with control cultures, which can grow at a different rate. Further studies on hyaluronic acid synthesis independently of protein synthesis were therefore necessary in osteogenesis imperfecta. Results of the present experiments confirm the decrease in collagen synthesis. In addition, the increase in hyaluronic acid synthesis was also noted when the calculations were carried out per unit of DNA in the cultures. The molecular-weight dispersion of hyaluronic acid in OI fibroblast culture medium is described.

Materials and methods

Cells

Fibroblasts were cultured from skin biopsies of patients and age-matched controls (Table 1) in Dulbecco's modification of Eagle's medium supplemented with 15% (v/v) newborn-calf serum (both from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), 23 mM- NaHCO_3 , 20 mM-Hepes (Gibco-Biotech Ltd., Paisley, Renfrewshire, Scotland, U.K.), 100 units of G-penicillin/ml and 50 μg of streptomycin sulphate (Orion Oy Ltd., Helsinki, Finland)/ml. The cultures were monitored for mycoplasma contamination with DNA/fluorochrome Hoechst 33258 staining (Chen, 1977).

Abbreviations used: OI, osteogenesis imperfecta; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Labelling of cultures with [^3H]proline

Confluent cultures were labelled for 48 h with L-[5- ^3H]proline (3.3 $\mu\text{Ci/ml}$; specific radioactivity 15.3 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) in Dulbecco's medium lacking glutamine and serum but supplemented with sodium ascorbate (50 $\mu\text{g/ml}$) and β -aminopropionitrile fumarate (50 $\mu\text{g/ml}$) (both from Sigma Chemical Co., St. Louis, MO, U.S.A.). After the labelling period the proteins in the media were precipitated and washed with 80% (v/v) ethanol. The cell layers were scraped off into 0.9% NaCl, duplicate samples were taken for protein determination (Lowry *et al.*, 1951), and the residue was precipitated and washed with 80% ethanol. Total and hydroxyproline radioactivities of the hydrolysed (3 h, 130°C, 6 M-HCl) cell-layer precipitates were determined with the method of Juva & Prockop (1966).

In the pulse-chase experiments, confluent cultures of OI line 1 and foetal control were labelled for 2.5 h with 10 μCi of [^3H]proline/ml. After aspirating the medium, the cell layer was rinsed once with 0.9% NaCl. A fresh culture medium without radiolabel was added and the cultures were incubated for 0, 45, 90 or 190 min. The media were separated, and the cell layers were rinsed twice with 0.9% NaCl, which was combined with the media. Phenylmethanesulphonyl fluoride and *p*-chloromercuribenzoate (both 200 μg), L-proline (final concn. 0.1%) and EDTA (final concn. 0.02%) were added and the media were dialysed against distilled water. The cell layers were scraped off into 0.9% NaCl, duplicate samples were taken for protein determination (Lowry *et al.*, 1951) and the residue was dialysed with phenylmethanesulphonyl fluoride, *p*-chloromercuribenzoate, L-proline and EDTA. Total and hydroxyproline radioactivities of the dialysed media and cell layers were determined after hydrolysis (3 h, 130°C, 6 M-HCl) (Juva & Prockop, 1966).

Labelling of cultures with [^3H]glucosamine

The cells were plated on 20 cm² plastic Petri dishes (Nunc Co., Roskilde, Denmark) to a density of 10 000 cells/cm². Synthesis of hyaluronic acid and sulphated glycosaminoglycans were studied in normal and OI skin fibroblast cultures at different growth stages. The cultures were exposed for 24 h to D-[1- ^3H]glucosamine hydrochloride (4.6 $\mu\text{Ci/ml}$; sp. radioactivity 3.2 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) in Dulbecco's medium lacking glutamine and serum 24 h after changing the growth medium. After labelling, the media and cell layers were separated.

The cell layers were scraped off and homogenized into 1.5 ml of phosphate-buffered saline (0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4), a 675 μl sample was taken for DNA determination (Jalkanen *et al.*, 1980), and duplicate 400 μl samples

from cell suspension and medium were taken for the separation of hyaluronic acid from sulphated glycosaminoglycans (Saarni & Tammi, 1977). The remaining medium (2.2 ml) was dialysed against 0.02 M-phosphate buffer, pH 7.4, at 4°C for gel filtration of the [^3H]glucosamine-labelled components in a column (1.5 cm $\times$ 35 cm) of Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden). The runs were carried out at room temperature in 0.02 M-phosphate buffer, pH 7.4, with a flow rate of 10 ml/h. Fractions (1.25 ml) were collected into the counting vials, to each of which was added 10 ml of scintillation fluid {15 g of PPO (2,5-diphenyloxazole), 50 mg of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene], 600 ml of toluene and 400 ml of nonylphenoxylethylene (Berol 09; Turun Saippua Oy Ltd., Turku, Finland)}. The radioactivities were counted in a Packard Tri-Carb model 3353 liquid-scintillation spectrometer (Packard Instrument Co., Downers Grove, IL, U.S.A.).

For the determination of the relative proportion of hyaluronic acid, glycoproteins and sulphated glycosaminoglycans in Sepharose 2B fractions, 1 ml of eluate (total volume 1.25 ml) was taken for the determination of the radioactivity, and the remaining 0.25 ml from fractions 14–23, 24–33, 34–43 and 44–53 were pooled and freeze-dried. Glycoproteins, hyaluronic acid and sulphated glycosaminoglycans were separated by the method of Saarni & Tammi (1977).

Results

Protein synthesis

It was shown previously (Turakainen *et al.*, 1980; Krieg *et al.*, 1981) that OI fibroblast cultures show cell-line-specific differences in the ratio of type III to type I collagens. Table 1 shows the relative synthesis of type I and type III collagens by OI fibroblasts of the present study determined from the CM-cellulose chromatograms of [^3H]proline-labelled culture medium after limited pepsin digestion. The relative synthesis of type III collagen was nearly normal in one OI cell line (OI 3) of unknown OI type and increased in others (OI 1, 2 and 4) which represent OI type I or II (Sillence *et al.*, 1979).

To study the protein-synthesizing capacity of OI fibroblasts the OI and control cells were incubated with [^3H]proline in serum-free medium for 48 h. Peptide-bound total- ^3H and [^3H]hydroxyproline radioactivities in medium and cells were determined after precipitation of proteins with ethanol. Two OI cell lines (1 and 4) incorporated [^3H]proline into proteins at a normal rate, but OI lines 2 and 3 did so at half the normal rate (Fig. 1a). Collagen synthesis determined as incorporation of [^3H]proline into protein-bound [^3H]hydroxyproline was decreased in

Table 1. *Source of cells*

Relative-synthesis values are calculated from the radioactivities in the carboxymethyl-cellulose chromatograms of [³H]proline-labelled culture media after limited pepsin digestion (Turakainen *et al.*, 1980). Abbreviations used: F, female; M, male; OI, osteogenesis imperfecta; A.T.C.C., American Type Culture Collection, Rockville, MD, U.S.A.

Cell line	Sex	Diagnosis	Relative synthesis of collagen types (%)	
			Type I	Type III
Control				
Foetal		Normal skin (20 weeks of gestation)	85	15
Newborn	F	Normal skin	92	8
11 years	M	Normal skin	82	18
45 years	M	Normal skin	80	20
OI				
Newborn (OI 1)	F	A.T.C.C. 1262. OI type II*	51	49
9 years (OI 2)	F	OI type I*	55	45
10 years (OI 3)	M	OI type ?	77	23
45 years (OI 4)	F	OI type I*	58	42

* Classification of Silience *et al.* (1979).

Fig. 1. Incorporation of [³H]proline into (a) protein and (b) collagen, and (c) the relative proportion [(b)/(a)] of collagen in OI and control (C) skin fibroblast cultures

Confluent cultures were labelled with [³H]proline. Proteins of medium and cell-layer samples were precipitated and washed with 80% (v/v) ethanol. Total ³H and [³H]hydroxyproline radioactivities of the hydrolysed precipitates were determined (Juva & Prockop, 1966). The results are expressed as d.p.m./μg of cell-layer protein ± s.e.m. The number of replicate samples is given in each column (n).

all four OI cell lines studied here (Fig. 1b), as shown previously for OI line 1 (A.T.C.C. 1262) (Steinmann *et al.*, 1979; Barsh & Byers, 1981) and for several cell lines representing OI type I (Barsh &

Byers, 1981). Furthermore, in OI lines 2 and 3, collagen synthesis was decreased more than the synthesis of other proteins, as shown by the decreased collagen/total protein ratio in Fig. 1(c).

Glycosaminoglycan synthesis

Effect of donor age and culture density. The radioactive macromolecules of control cultures were digested with papain after 24h labelling with [^3H]glucosamine. Glycosaminoglycans were precipitated on to cellulose ester filter membranes. Hyaluronic acid was separated from sulphated glycosaminoglycans by selective elution with HCl. Hyaluronic acid synthesis was highest at the exponential phase of growth in all cell lines and decreased markedly when the cultures became confluent (Fig. 2a). Hyaluronic acid synthesis was highest in fibroblasts cultured from a foetus (20 weeks of gestation) $[(15-72) \times 10^3 \text{ d.p.m./}\mu\text{g of DNA}]$ and at a lower level $[(6-32) \times 10^3 \text{ d.p.m./}\mu\text{g of DNA}]$ in fibroblasts from a newborn control (40–70% of that of foetal fibroblasts at the same cell density) (Fig. 2a). Skin fibroblasts from an 11-year-old and those from a 45-year-old control person produced even less (5–42 d.p.m./ $\mu\text{g of DNA}$; 25–58% of the values given by foetal cells at the same cell density).

The synthesis of sulphated glycosaminoglycans was even more dependent on the age of the fibroblast donor, but not as dependent on the cell density as the synthesis of hyaluronic acid (Fig. 2b). The values were highest in foetal skin fibroblasts $[(14-26) \times 10^3 \text{ d.p.m./}\mu\text{g of DNA}]$ and markedly less in newborn skin fibroblasts $[(4-11) \times 10^3 \text{ d.p.m./}\mu\text{g of}$

DNA; 25–45% of foetal-fibroblast values]. Cultures from an 11-year-old or a 45-year-old control person showed sulphated-glycosaminoglycan synthesis of only $(2-6) \times 10^3 \text{ d.p.m./}\mu\text{g of DNA}$ (19–25% of the amount produced by foetal fibroblasts). These results underline the importance of strict age and cell-density matching of control cultures in studies of glycosaminoglycan synthesis.

Synthesis of glycosaminoglycans in OI fibroblasts. As in the control cell lines, the OI lines synthesized hyaluronic acid maximally in the exponential phase of growth; the synthesis of sulphated glycosaminoglycans was not much dependent on the growth phase (result not shown). Hyaluronic acid synthesis (d.p.m./ $\mu\text{g of DNA}$) in OI cells was 1.1–4.5-fold higher than in age-matched controls in the exponential growth phase, and the synthesis of sulphated glycosaminoglycans was at the normal level. Radioactivities incorporated into glycosaminoglycans varied markedly in different experiments (Table 2). However, the ratio of radioactivities incorporated into hyaluronic acid to that into sulphated glycosaminoglycans remained always markedly higher in OI cultures than in control cultures, especially in the actively growing phase (0.7–3.2 in the controls and 4.1–7.5 in the OI cell lines; Fig. 3).

The distribution of glycosaminoglycans between culture medium and cell layer in OI cultures did not

Fig. 2. Incorporation of [^3H]glucosamine into (a) hyaluronic acid and (b) sulphated glycosaminoglycans by cultured control skin fibroblasts

The cultures were labelled in different growth phases with [^3H]glucosamine. Hyaluronic acid and sulphated-glycosaminoglycan radioactivities in the media and cell layers were determined (Saarni & Tammi, 1977). The results are the means of duplicate samples. ●, Foetal; ▲, newborn; ■, 11 years; ◆, 45 years.

Table 2. Incorporation of [³H]glucosamine into hyaluronic acid (HA) and sulphated glycosaminoglycans (sGAG) by OI and control skin fibroblasts in two different experiments

The cultures were labelled in the exponential growth phase with [³H]glucosamine (4.6 μCi/ml) for 24 h. Hyaluronic acid and sulphated glycosaminoglycan radioactivities in the media and cell layers were determined (Saarni & Tammi, 1977). The results are the means for duplicate samples.

		DNA (μg/dish)	HA (d.p.m./μg of DNA)	sGAG (d.p.m./μg of DNA)
Expt. 1	Control			
	Foetal	5.64	46 630	14 212
	Newborn	5.73	44 564	26 118
	11.5 years	5.38	5707	2324
	45 years	5.29	5070	890
	OI			
	9 years	4.36	20 000	2021
	10 years	5.58	25 707	2271
	45 years	4.29	22 405	1843
Expt. 2	Control			
	Foetal	8.91	17 559	14 243
	Newborn	4.51	21 930	6834
	11.5 years	5.86	10 634	2867
	45 years	3.66	17 235	4506
	OI			
	Newborn	2.07	64 950	9826
	9 years	4.64	19 129	2280
	10 years	3.42	22 452	3335
	45 years	4.51	18 638	2119

Fig. 3. Relative synthesis of hyaluronic acid (HA) and sulphated glycosaminoglycans (sGAG) in OI and control skin fibroblast cultures at different cell densities. Controls (closed symbols): ●, foetal; ▲, newborn; ■, 11 years; ◆, 45 years. OI (open symbols): ▽, 9 years; □, 10 years; ◇, 45 years.

differ from that in control cultures (results not shown). Thus we conclude that there is no defect in the secretion of glycosaminoglycans.

Molecular-weight distribution of [³H]glucosamine-labelled macromolecules. Gel filtration in a Sepharose 2B column was used to determine the molecular-weight distribution of medium [³H]glucosamine-labelled macromolecular complexes of control and OI cell cultures. Fig. 4 shows some elution profiles of dialysed control and OI culture (OI line 2) media. High-molecular-weight ($\geq 2 \times 10^6$) fractions were mostly hyaluronic acid (53–77% of total radioactivity) and low-molecular-weight ($\leq 10^5$) fractions mostly glycoproteins (72–97% of total radioactivity). Sulphated glycosaminoglycans were eluted in all fractions. The profiles were different at different cellular densities of the cultures. In the exponential phase the high-molecular-weight fractions predominated (Fig. 4, upper panels). When the cultures became confluent, the area of the high-molecular-weight region clearly decreased, indicating a relatively decreased proportion (and decreased synthesis) of hyaluronic acid (Fig. 4, lower panels). In the profiles of OI culture media the high-molecular-weight peak was 15–30% larger than in the controls.

Effect of hyaluronidase on procollagen secretion. A high concentration of hyaluronic acid in the culture medium impairs the secretion of procollagen, possibly by its interaction with plasma membrane (Ivaska, 1981). We tested the rate of procollagen secretion in the OI cell line in which hyaluronic acid synthesis is highest and procollagen secretion is lowest of our OI fibroblasts (A.T.C.C. 1262) in the presence or absence of testicular hyaluronidase. Protein-bound [³H]hydroxyproline radioactivities of the culture media and cell layers were determined after chasing the pulse-labelled cultures for up to 5 h. Addition of hyaluronidase to medium did not change the rate of procollagen secretion at the concentration that during 5 h completely destroyed the high-molecular-weight hyaluronic acid (result not shown).

Discussion

Previously (Turakainen *et al.*, 1980) we reported that the activities of the hyaluronate synthetase enzyme complex were 1.3–2.0-fold higher and the amount of radioactive hyaluric acid in [³H]glucosamine labelling experiments approximately 2-fold higher in skin fibroblasts cultured from forearm

Fig. 4. Molecular-weight distribution of [^3H]glucosamine-labelled macromolecules in OI (right) and control (left) fibroblast culture media

Cultures were labelled in different growth phases with [^3H]glucosamine. Culture media were dialysed against 0.02 M-phosphate buffer, pH 7.4, and fractionated on a Sepharose 2B column. Fraction 16 corresponds to the exclusion volume and fraction 50 to the inclusion volume of the column. The amount of DNA in the culture and the relative proportions of hyaluronic acid (HA), sulphated glycosaminoglycans (sGAG) and glycoproteins (GP) in pooled fractions are shown in each panel.

biopsies of OI patients than in skin fibroblasts cultured from control. Hyaluronic acid synthesis was compared with protein synthesis by using labelling of hyaluronic acid with [^3H]glucosamine and of proteins with [^{14}C]glycine. The observation of decreased synthesis of collagen in several OI cell lines made it necessary to re-examine the hyaluronic acid production per cell.

Comparison between the level of glycosaminoglycan synthesis in different cell lines requires caution, since, e.g., cell density, duration between medium changes or the activity of growth factors in serum all influence hyaluronic acid synthesis (Kurtz & Stidworthy, 1975; Hopwood & Dorfman, 1979; Ninomiya & Nagai, 1979; Hronowski & Anastasiades, 1980). In the present work, culture homogeneity and labelling conditions were optimized by changing the growth medium every day and exposing the [^3H]glucosamine label in serum-free medium 24 h after the last medium change.

Studies on hyaluronic acid and chondroitin sulphate (the main sulphated glycosaminoglycan

made by most fibroblasts) syntheses in several rat fibroblast cell lines were carried out by using chemical determination of various glycosaminoglycan fractions (Hronowski & Anastasiades, 1980). Like ourselves, those authors observed linear kinetics on a semilogarithmic scale for hyaluronic acid synthesis and on the linear scale for chondroitin sulphate synthesis in respect to increasing cell density. In our experiments a marked age effect in normal fibroblast cultures was also noticed, especially in the synthesis of sulphated glycosaminoglycans. It seems that there is a donor-age-dependent decrease in the synthesis of proteoglycan core protein.

Hyaluronic acid and proteoglycan syntheses in OI cells were re-estimated by using DNA determination as a reference. The results obtained by a cetylpyridinium chloride precipitation method and by Sepharose 2B elution profiles clearly show that the differences in hyaluronic acid synthesis in OI are real and significant. The incorporation of [^3H]glucosamine into glycosaminoglycans was altered so

that hyaluronic acid/sulphated glycosaminoglycan ratio was higher in all OI cultures than in control cultures.

A gradual decline in the synthesis of glycosaminoglycans has been shown to occur during the last passages of human fibroblast cultures before phase-out (Schachtschabel & Wever, 1978). The passage numbers used in our experiments were 2–9 for the controls and 6–13 for the OI cell lines, which does not explain the differences in glycosaminoglycan synthesis between OI and control cells.

It has been shown in our laboratory (Vuorio *et al.*, 1982) and elsewhere (Castor & Dorstewitz, 1966) that synovial fibroblasts from rheumatoid-arthritis or other synovitis patients produce hyaluronic acid (often in increased amounts) which has a decreased proportion of the high-molecular-weight ($\geq 10^6$) fraction. Thus it was considered worthwhile to study the molecular weight of hyaluronic acid complexes in OI cultures. The Sepharose 2B elution profiles of media showed that the high-molecular-weight peaks in the OI specimens were 15–30% larger than those of controls. However, the cell density had a marked effect on the profiles; high density decreased the high-molecular-weight fraction in both samples. The molecular weight of hyaluronic acid was also shown to be normal in skin fibroblasts cultured from Marfan-syndrome patients (L. Blåfield, unpublished work) in which increased synthesis of hyaluronic acid has been documented (Matalon & Dorfman, 1968).

Several other authors have reported alterations in glycosaminoglycans in OI (Spencer, 1962; Solheim, 1969; Bleckmann *et al.*, 1971; Engfeldt & Hjerpe, 1976; Cetta *et al.*, 1977). An increase in the total amounts of glycosaminoglycans in OI bone and cartilage (Spencer, 1962; Bleckmann *et al.*, 1971) and in their sulphation (Engfeldt & Hjerpe, 1976) has been reported. However, a low chondroitin sulphate content has been recorded in some bone and cartilage specimens (Solheim, 1969; Cetta *et al.*, 1977). In addition, glycosaminoglycan excretion in urine has been concluded to be normal in OI patients (Berggren *et al.*, 1969).

Large amounts of hyaluronic acid are present in embryonic skin, whereas adult skin contains more chondroitin sulphate than hyaluronic acid (Lehtonen, 1968). Histological sections of tissues from OI patients show various signs of immaturity (see, e.g., McKusick, 1972). A high level of synthesis of hyaluronic acid and type III collagen in many skin fibroblast cultures of OI patients might be linked together and indicate that the fibroblasts have not reached their final differentiation stage. Various individual defects in the type-I-collagen gene in OI might lead to defects in synthesis or in biochemical properties of type I collagen and to compensation with type III collagen. The abnormally high pro-

portion of type III collagen then decreases the biological competence of skin and bone (Müller *et al.*, 1977).

OI is heterogeneous and has been classified into four clinical varieties, which also have subgroups (Sillence *et al.*, 1979). Several structural mutations in type I collagen have already been revealed (Peltonen *et al.*, 1980; Barsh & Byers, 1981; Steinmann *et al.*, 1983). The present work points to an elevated synthesis of hyaluronic acid in OI fibroblast cultures, a finding which has been detected in the Marfan (Matalon & Dorfman, 1968) and suggested in the Hurler (Germinario *et al.*, 1973) syndromes, all of which are severe heritable connective-tissue disorders. Elevated hyaluronic acid synthesis is not diagnostic for OI, but only indicates a general disorder of connective tissue. Its usefulness in, e.g., prenatal diagnosis, has not been attempted. In most cases of OI the precise chemical defect has not yet been demonstrated, and once detected, its investigation may be too laborious for clinical purposes. If the family history is positive for OI, a high (hyaluronic acid)/(sulphated glycosaminoglycan) synthesis ratio in cultured amniotic-fluid cells should be useful and point to an affected child.

I gratefully acknowledge the valuable suggestions and comments of Associate Professor Risto Penttinen and the expert technical assistance of Mrs. Eina Samstén and Mrs. Liisa Peltonen. This work was supported in part by an institutional grant from the Medical Research Council of the Academy of Finland and by a personal grant from the Emil Aaltonen Foundation.

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