CELL SURFACE CHANGES IN SENESCENT AND WERNER'S SYNDROME FIBROBLASTS: THEIR ROLE IN CELL PROLIFERATION

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INTRODUCTION

Werner's syndrome (WS) has segmental progeroid features and pedigree analysis suggests its autosomal recessive inheritance (Epstein et al., 1966). Two of the clinical characteristics of Werner's syndrome are hyperkeratinized skin and an excessive excretion of hyaluronic acid into the urine (Tokuraga et al., 1975, Goto and Murata, 1978) suggesting some disorder(s) of connective-tissue metabolism. Cultured fibroblasts from human skin have been used for characterization of genetic defects in many inherited diseases. Epstein et al. (1966) cultured fibroblast-like cells from the skin of patients with WS and noted that they grew very poorly. Since then, WS fibroblasts have been used to find the explanation for the shortened lifespan in vivo and in vitro (Martin et al., 1970). However, the primary molecular defect that retards cell growth has remained elusive. Recently, Salk (1982) published an extensive review article summarizing 15 years of research since that of Epstein et al.

Cell surface is known to have some role in the regulation of cell proliferation and cell communication through interaction with adjacent cell surfaces, with the intercellular matrix, or both (Matuoka and Mitsui, 1981b). Glycosaminoglycans (GAGs), such as hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparan sulfate, make up the cell-surface peripheral coat and give a negative charge on the cell surface; they are excreted into the extracellular space, changing the environment for the neighboring cells. Thus, any altered GAGs produced by fibroblasts may play a critical role in cell communication and cell proliferation.

We report here alterations of cell-surface GAGs in conjunction with changes in surface (negative) charge and cell proliferation capacity in fibroblasts from young and old patients with and without Werner's syndrome.

MATERIALS AND METHODS

Cell strains and cell cultures

The cell strains used were TIG-1 human fetal lung fibroblasts (Mitsui et al., 1980, Ohashi et al., 1980), GRC-series skin fibroblasts (Schneider and Mitsui, 1976) from 13 men (aged 0-89 years), and WS-series skin fibroblasts from 5 male patients aged (1961 years) with WS. WS fibroblats WS TY01 and WS TY02 at 5 population doubling levels (PDL) were a gift from Matsumura (Matsumura et al., 1980) and WS 12KO at 6 PDL from Fujiwara (Fujiwara et al., 1977). Primary cultures of the other skin fibroblasts were obtained by outgrowth as described before (Schneider and Mitsui, 1976; 1978). The cells were cultured in Eagle's minimum essential medium containing nonessential amino acids, supplemented with 10% fetal bovine serum, in an environment of 5% CO_2 and 95% air by 37°C. A routine subcultivation was achieved by harvesting cells with 0.25% trypsin solution in CA^{++}, Mg^{++}-free phosphate buffered saline [PBS(-)]; the cells were inoculated at 1:4 or 1:2 split ratio.

Cell elecrophoresis

Confluent cells, 99% of which were in the G_1 phase of cell growth as determined with a cytofluorometer, were rinsed and dispersed with 0.05% EDTA containing PBS (-) solution. The cells were centrifuged and resuspended in M/15 phosphate buffer containing 5.4% glucose (pH 7.30). The electrophoretic mobility (EPM) of each cell suspended in this buffer was measured using Cell Electrophoresis Microscope Systems (Sugiura Laboratory). A mean value and standard deviation were calculated from the EPM of 50-100 cells. The values were expressed as μm/sec/V/cm. EPM

reflects a negative charge density on a unit area of cell surface within a depth of about 10 Å.

Radioisotope Incorporation

Confluent cells were cultured in medium containing D-^{3}H glucosamine hydrochloride (specific radioactivity 38 Ci/mmole, 5 μCi/ml x 10 ml) under normal conditions for 48 hours. After being washed, the cell layer was treated with 0.25% trypsin solution for 30 minutes to isolate the cell-surface GAGs fractions.

Isolation of GAGs and Two-Dimensional Electrophoresis Analysis

Cell-surface GAGs were precipitated by cetylpyridinium chloride after removal of the protein fraction and the purified cellulose acetate membrane. For quantitative determination, each spot on the mebrane was cut off and transferred to a scintillation vial to measure the radioactivity in each glycosaminoglycan. Details were described in a previous paper (Matouka and Mitsui, 1981a).

Cell Culture on the Fixed Cell Sheets with Enzyme Treatment

Confluent cells were fixed with 2.5% glutaraldehyde for 30 minutes. After thorough washing and incubation in serum-free medium overnight, the cell layer was treated with *Streptomyces* hyaluronidase (0.03 TRU per cm^2) at 50°C, chondroitinase ABC (0.0015 units per cm^2) at 37°C, or *Flavobacterium* heparitinase (87 units/mg, 11 μg per cm^2) at 43°C for 4 hours. Nitrous acid (0.2 ml per cm^2, as 18% $NaNO_2$ aqueous solution) was also used to remove heparan sulfate. The details were described before (Matuoka and Mitsui, 1981b). To see the effect of cell sheets on the fibroblast proliferation, fibroblasts were inoculated into dishes with the fixed cell sheets on which the GAGs were treated with degradation enzymes.

RESULTS

Cell Growth

Fibroblast-like cells were obtained by outgrowth from skin fragments of normal donors and patients with WS. Although migration and outgrowth of fibroblasts were very slow from WS skin fragments, selective outgrowth of keratinocytes was sometimes discernible. Serial cultivations of fibroblasts were performed at 1:4 or 1:2 split ratio to the end of their lifespans in vitro. A typical cell-growth characteristic of WS fibroblasts is seen in Fig. 1. The proliferative capacity of WS fibroblasts

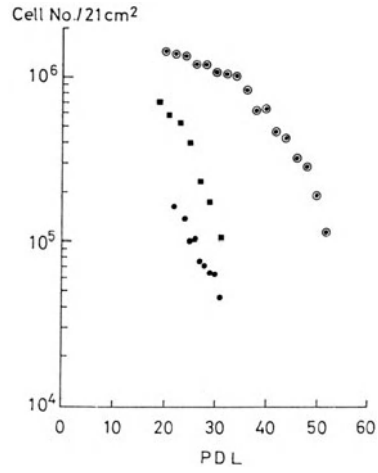

Fig. 1. Cell number at harvesting time throughout life span of normal and Werner fibroblasts. Cell number density at confluent was counted on the 7th day after the inoculation at 1:4 or 1:2 split ratio. Skin fibroblasts from normal subjects of 56 years old (⊙) and 88 years old (■), and skin fibroblasts from a Werner syndrome patient of 56 years old (●).

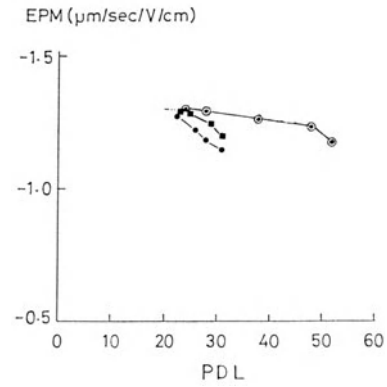

Fig. 2. Decrease in electrophoretic mobility (EPM) during in vitro aging. Confluent state of cell at each harvesting time (Fig. 1) was dispersed with 0.05% EDTA solution and EPM of 50-100 cells were measured by Cell Electrophoresis Microscope System. The cells used are of the same origins as shown in Fig. 1.

was markedly less than that of the age and sex-matched normal control fibroblasts. An increase in cell size, characteristic of senescent cultures (Mitsui and Schneider, 1976a), was also evident in the WS fibroblasts.

Electrophoretic Mobility (EPM)

At each time of cell harvesting (Fig. 1), usually one week after the subcultivation, confluent cells in the parallel cultures were dispersed in 0.05% EDTA solution without using enzymes and subjected to cell electrophoresis. Cell EPM was found to decrease with passage number in vitro (Fig. 2). On the other hand, cell-size distribution has been found to increase and to become more heterogenous with aging in vitro (Mitsui and Schneider 1976a,b,c) and EPM, which reflects the amount of negative charge per unit area of cell-membrane surface, declines when cells are enlarged by hypotonic treatment. Determination of the EPM of different size cells in the young and senescent cultures confirmed that a decrease in charge density noted in senescent cultures occurred even in the small-cell populations of heterogeneous senescent cultures.

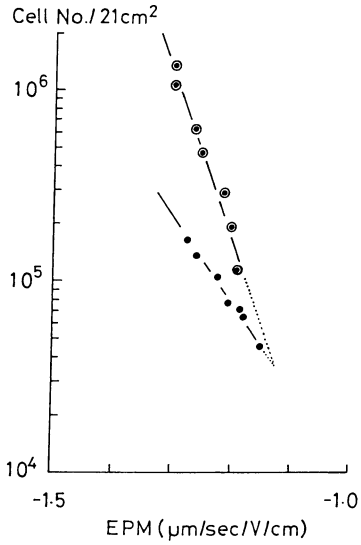

Fig. 3. Relationship between confluent cell number and electrophoretic mobility at each passage. As shown in Fig. 1 and 2, cell number and EPM at harvesting time of each passage declined with aging. Their linear relationships were confirmed in all the cell strains examined. Skin fibroblasts from 56-year-old normal (⊙) and Werner syndrome (●) subjects.

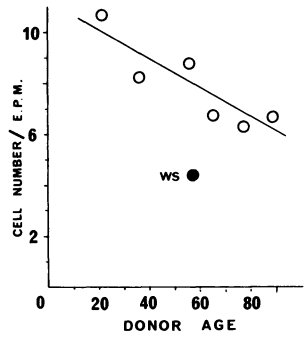

Fig. 4. Donor age effect on slope of linear regression between cell number and EPM. Slope of line in Fig. 3 and other similar data were plotted as a function of donor age. (○): skin fibroblasts from different ages of normal donors. (●): cell from a Werner syndrome patient.

When EPM was plotted as a function of numerical density at harvesting time throughout the life span, a strict linear relationship between them was found in all the cell strains examined. Figure 3 demonstrates two typical examples of these linear relations in WS and normal control fibroblasts and suggests a decline in the slope in WS fibroblasts. When slopes were plotted for fibroblasts from donors of different ages against donor age, an age-related decline in slope was found (Fig. 4). The same figure also suggests that the patient with WS had older characteristics for his age in this index.

Haemadsorption Capacity in WS Fibroblasts

When Concanavalin A-coated red blood cells were adsorbed onto the fibroblast cell surface (Fig. 5), the amount of red blood cells per unit area of fibroblast surface increased in vitro with cellular aging (Aizawa and Mitsui, 1979). Also, the increase in the haemadosorption capacity of the fibroblasts was found to reflect a decrease in the cell-surface negative charge on the senescent cells (Aizawa et al., 1980a). Examination of the haemadosorption capacity of fibroblasts from normal subjects (13 men) and WS patients (5 men)(Fig.6) suggested that WS patients have older characteristics for their age in this index (Aizawa et al., 1980b). This increase in haemadsorption capacity in WS cells was well correlated with the above-mentioned decrease in the slope of EPM per cell, suggesting a decrease in the density of the negative charges on the cell surface.

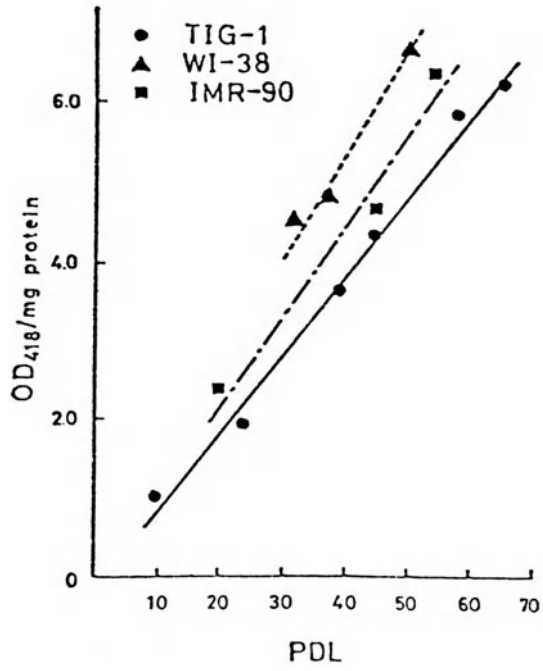

Fig. 5. Adsorption of Con-A treated red blood cells on cell surface of senescent fibroblasts. Note the linear increases in haemadsorption activity with cellular aging of WI-38, IMR-90 and TIG-1 cells.

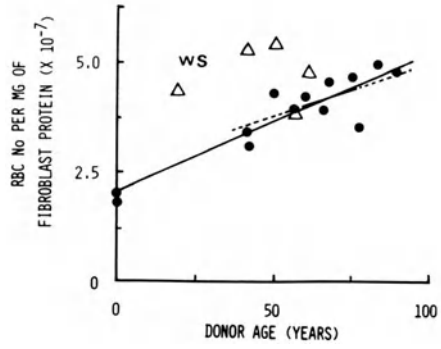

Fig. 6. Haemadsorption activity of fibroblasts as a function of donor age. Skin fibroblasts (20 PDL) from normal subjects (●; 0-88 years old) and fibroblasts from Werner patients (△; 29-56 years old) were examined for the Con-A treated red blood cell adsorption capacity. Data from Aizawa et al. (1980b) were redrawn.

Role of Cell-Surface Glycosaminoglycans (GAGs) and Sialic Acid in Electrophoretic Mobility

Negative charges on the cell surface are thought to be due mainly to the sialic acid and sulfate in the GAGs in a thin layer (10 Å) of the cell-surface coat. In order to know the contribution of each GAG and sialic acid on age-related changes in negative charge, EPMs of young and senescent cells were examined after treatment of the cell surface with various degradation enzymes. Summarized data are shown in Table 1. A decrease in EPM after treatment with neuraminidase, hyaluronidase, chondroitinase ABC, or heparitinase indicates the contribution in negative charge of neuraminic acid, hyaluronic acid, chondroitin sulfates and heparan sulfate, respectively. It is apparent that the degree of contribution of each anion changes with aging. In early passaged cells, hyaluronic acid had the greatest contribution (28% of the total negative charge), but only a small contribution (12%) in late passaged cells. During cell aging in vitro, a definite decrease in negative charge was observed (1.242 - 1.658 = -.416). During the same period, hyaluronic acid contents of the surface coat decreased markedly (.149 - .471 = -.322), chondroitin sulfates mildly (.087 - .288 = -.201), and neuraminic acid slightly (.206 - .297 = -.081). On the other hand, heparan sulfate seems not to have decreased but rather to have increased slightly.

Thus, it is noteworthy that the age-related decrease in the negative charge in vitro would be due to mainly a decrease in hyaluronic acid and that the proportion of heparan sulfate became greatest (31%) in senescent cells.

Glycosaminoglycans on Cell Surface Coat

Human diploid fibroblasts produce hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and heparan sulfate, which are excreted mostly into medium (Matuoka and Mitsui, 1981a). The amount of each GAG on the cell surface, however, can be examined by prolonged incubation with labeled precursors. Glycosaminoglycans on the cell surface at the confluent state of growth were released by treatment with trypsin after incubation for 48 hours in a medium containing ^{3}H-glucosamine. The purified GAGs were subjected to two-dimensional electrophoresis to quantitate each fraction of GAGs. Figure 7 demonstrates the amounts of radioactivity in hyaluronic acid (HA), chondroitin sulfates plus dermatan sulfate (CS) and heparan sulfate (HS) per mg protein in young, middle, and late passage cells. It is quite clear that with cell aging in vitro hyaluronic acid and chondroitin sulfates dramatically decline and that heparan sulfate gradually increases during the same period. Thus, heparan sulfate becomes a main component of GAGs in the

Table 1

Electrophoretic Mobility
Decrease in Senescent Cells and Role of GAGs

(μm/sec/V/cm)

	TREATMENT			
PDL Control	Neuraminidase	Hyaluronidase	Chondroitinase	Heparitinase
15 -1.658 ±.108	-1.361 ±.103 -.297*	-1.187 ±.106 -.471*	-1.371 ±.128 -.288*	
(25 -1.605)				-1.218 ± .079 -.387
65 -1.242 ± .113	-1.036 ± .138 -.206*	-1.093 ± .130 -.149*	-1.135 ± .137 -.087*	-0.851 ± .059 -.391*
Diff. -.416**	-.081**	-.322**	-.201**	+.004*

* Difference in EPM between control and enzyme-treated cells at each passage.

** Difference in EPM between young (15 PDL) cells and old (65 PDL) cells in control and enzyme-treated populations.

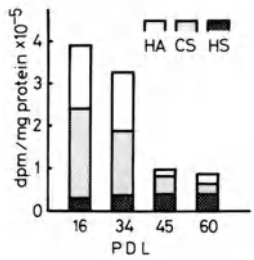

Fig. 7. Changes in glycosaminoglycans on the cell surface with in vitro cellular aging. 48-hour incorporation of ^{3}H-glucosamine into hyaluronic acid (HA), chondritin sulfate (CS) and heparan sulfate (HS) on the cell surface examined as a function of in vitro passage of TIG-1 fibroblasts.

cell-surface coat of the senescent cells. These findings correlate with the observations obtained from measuring the enzyme-treated cell EPM.

Retardation of Cell Growth on the Fixed Cell Surface Sheets

The numerical saturation density in vitro of cells at the confluent stage of growth declines steadily with age, in spite of repeated changes of medium. Because contact inhibition of cell growth in normal fibroblasts is thought to be mediated by cell-surface interaction (Dulbecco et al., 1970) and age-related cell surface changes have been found, we pursued the possibility that the cell-surface constituents, such as glycosaminoglycans, contributed to the regulation of cell proliferation.

To find out the effect of direct contact of the cell surface, avoiding metabolic communication, we treated the cells with fixatives and made cell-surface sheets onto which another cell strain was inoculated and left to grow. Glutaraldehyde was the best fixative, seemingly preserving the natural configuration of cell-surface coat as described before (Matuoka and Mitsui, 1981b). Cell growth and saturation density were lower when the cells were inoculated onto fixed cell-surface sheets. As seen in Table 2, a greater inhibition of cell growth was observed when the cells were inoculated onto the cell sheet surface from older cultures. Increased synthesis and accumulation of heparan sulfate on the cell surfaces of senescent cultures were accompanied by an enhanced inhibitory effect on cell growth (Figure 7 and Table 2).

Table 2

Heparan sulfate content and growth-inhibitory effect of fixed cell-surface sheets: Changes with aging.

Age (PDL)	HS contents (pg / cell)	Age (PDL)	Growth inhibition (%)
17	1.5	12	16
38	1.8	31	35
55	2.5	47	45

Note that senescent fibroblasts accumulated more heparan sulfate (HS) on the cell surface and experienced greater growth-inhibition.

To confirm the role of heparan sulfate in cell growth regulation, we treated the fixed cell sheet surface with GAG degradation enzymes or agents and examined their effect on recovery from growth inhibition. Table 3 demonstrates that cell-surface chondroitin sulfates had no contribution on the observed cell-growth inhibition, and that removal of heparan sulfate completely restored cell growth. Although hyaluronidase treatment resulted in 50% recovery of cell growth, it removed about 6% of the heparan sulfate in addition to 75% of the hyaluronic acid. As the most superficial layer of the cell surface is more easily removed, hyaluronidase may have removed the most effective part of the heparan sulfate for the cell-growth manipulation. Since the amount of hyaluronic acid on cell surface became small and relative amount of heparan sulfate became very high in senescent cells (Fig. 7), it is evident that heparan sulfate has the most effective role in inhibiting the cell growth on senescent cultures. The cell-growth inhibiting effects of fixed cell surface coats from WS fibroblasts and from age-matched normal fibroblasts were compared. As seen in Figure 8, WS fibroblast surface coat was found to have the greater inhibitory effect on cell growth.

Examination of ^{3}H-glucosamine incorporation into WS fibroblasts and normal fibroblasts from donors of different ages confirmed that the total amount of heparan sulfate on the cell surface was higher in WS fibroblasts (Table 4).

Thus, we conclude that there is a possibility that changes in cell-surface GAGs may be a cause of the retarded cell growth of WS cells and also of senescent fibroblasts.

DISCUSSION

Although cellular changes at the nuclear level have been examined in WS fibroblasts as well as senescent fibroblasts in vitro (Mitsui and Schneider, 1976a,b,c; Fujiwara et al., 1977; Mitsui et al., 1979, 1980a,b, 1981; Takeuchi et al., 1982; Ide et al., 1983; Mitsui and Sakagami, 1983), cell-surface changes also should be regarded as possible primary causes of changes in cell-proliferation capacity. There have been several reports of cell-surface (membrane) defects in WS fibroblasts. These include reduced expression of HLA antigens (Goldstein and Singal, 1974), increased procoagulant activity of tissue factor (Goldstein and Niewiarowski, 1976), resistance to insulin (Nakao et al., 1978,

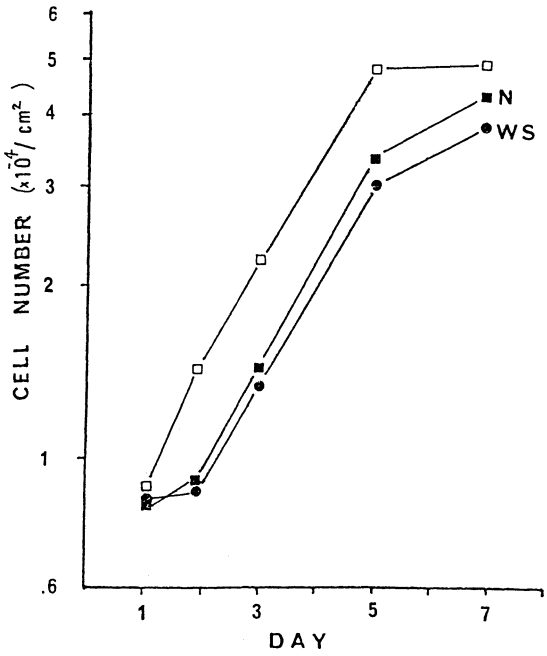

Fig. 8. Cell growth on the fixed cell sheets of normal and Werner fibroblasts. Fixed cell sheets were prepared from Werner fibroblasts (●, 5 PDL) and donor age-matched normal fibroblasts (■, 25 PDL). Normal cells were grown on the fixed cell sheets.

Table 3

Cell Growth Recovery After Removal of GAGs

	Removal of GAGs	Recovery of Cell Growth
Normal Control		(100%)
Treatment		
Chondroitinase ABC	78% (CS)	-7%
Hyaluronidase	75% (HA), 6% (HS)	50%
Heparitinase	75% (HS)	95%
Nitrous Acid	88% (HS)	100%

Fixed cell sheets were treated with enzymes to remove GAGs and tested for cell growth recovery. Untreated cell sheets inhibited cell growth by 40%. When the enzyme-treated cell sheet lost the inhibitory effect completely, recovery of cell growth was assessed as 100%.

Smith et al., 1980) and a decrease in insulin receptors (Beadle et al., 1978). However, their relation to shortened replicative life span in WS fibroblasts has not been examined.

A decrease in total glycosaminoglycans production (Weber et al., 1980; Matuoka and Mitsui, 1981a), changes in hyaluronidase sensitivity (Yamamoto et al., 1977), and an increase in accumulation of heparan sulfate on cell surface (Matuoka and Mitsui, 1981a, b; Sluke et al., 1981) in senescent cultures have been reported, as has a marked increase in the accumulation of sulfated GAGs in Werner fibroblasts (Tajima et al., 1981; Salk, 1982). In our previous paper, we claimed that cell-surface heparan sulfate is involved in the density-dependent inhibition of cell proliferation in senescent fibroblasts (Matuoka and Mitsui, 1981b).

In the present study, we showed that WS cells had a poorer cell growth ability and a lowered negative-charge density as revealed by the retarded EPM. A strict linear relationship

Table 4

GLYCOSAMINOGLYCANS IN NORMAL AND WS FIBROBLASTS

Cell strain	Distribution of ^{3}H-GAGs (%)			Hep. Sulf. (%) in surface GAGs	Hyal. Acid in Medium dpm x 10^{-5}/dish
	Surface	Medium	Cell		
Normal					
29 years	6.5	73.8	19.7	21.3	1.60
56 years	6.1	72.6	21.2	42.5	2.91
88 years	3.7	71.7	24.6	54.5	5.26
WS					
19 years	13.9	74.0	12.2	34.6	6.90
29 years	5.8	82.3	11.9	33.0	9.47
46 years	12.6	74.8	12.6	37.9	6.64

between EPM and cell number at confluency in several passages was found in WS fibroblasts as well as in normal donor cells. The slope of this line (cell number change per EPM change) was lower in WS fibroblasts, depicting older characteristics for the donor age of the cells. This lower slope reflects a greater inhibition of cell growth in early-passage cultures compared with the changes in negative-charge density on surface coat, as evidenced by the other series of experiments. A lower negative-charge density in WS cells than in normal controls was confirmed by their increased red blood cell adsorption capacity. A decreased negative-charge density during the aging of normal lung fibroblasts in vitro was ascribed to decreases in the hyaluronic acid and chondroitin sulfates at the cell surface, as revealed by EPM changes after treatment with GAG degradation enzymes and by changes in the amount of GAGs produced. These experiments also demonstrated that heparan sulfate slightly increased with cell aging and became the main component of the cell surface coat. Involvement of cell-surface heparan sulfate in the lowered ability of senescent cells to proliferate is shown by the growth inhibition of cells inoculated onto the fixed cell-surface sheet with various heparan sulfate contents. WS fibroblasts had a greater inhibition effect than cells from an age-matched donor. Cell surface plays an important role in the regulation of function and cell growth through direct interaction with the cell surfaces or with the environment. Thus, changes in cell-surface properties should be extensively examined to discover the mechanisms of aging in WS and normal cells.

SUMMARY

Cell surface is known to participate in the regulation of cell proliferation through interaction with adjacent cell surfaces or the extracellular matrix, or both. A clinical survey of the Werner syndrome suggests some disorders in glycosaminoglycan metabolism. Also, the skin fibroblasts derived from the patients with WS have a reduced proliferation capacity. We here examined, in vitro and in vivo, alterations of the cell-surface properties of WS cells and aging human fibroblasts.

Cell-surface negative charges, examined by electrophoretic mobility of dispersed single cells in buffer, were seen to decline steadily as a function of cumulative population doublings. A strict linear relationship was found between electrophoretic mobility (μm/sec/V/cm) and number of cells harvested at each passage in all cell lines examined. The slope of this line in cells from donors of different ages indicated that WS fibroblasts resemble cells from much older normal controls. The same conclusion was drawn from our previous study of Con A-mediated red cell adsorption, which was confirmed as

reflecting an alteration of cell-surface coat negative charge. Electrophretic mobility after treatment of cell surface with degradative enzymes showed that the cell-surface negative charges were attributable to sialic acid, chondroitin sulphates, hyaluronic acid, and heparan sulphate. Two-dimensional electrophoresis of ^{3}H-glucosamine incorporated glycosaminoglycans (GAGs) revealed that heparan sulphate was the main component of GAGs on the fibroblast cell surface and that the relative amount of heparan sulphate among GAGs on the cell surface increased in vitro with the number of passages. Growth kinetics of fibroblasts on sheets of fixed cells treated with a fixative (glutaraldehyde) and degradative enzymes were examined to elucidate the role of cell-surface GAGs in the regulation of cell proliferation. Cell growth was inhibited 40% when the fibroblasts were cultured on the fixed sheets of late passage cells. Treatment of the fixed cell sheets with heparitinase or nitrous acid resulted in complete recovery from the growth inhibition. Cell growth on sheets of fixed cells derived from young, middle, and senescent fibroblasts showed that the surface of the senescent cells had the greatest inhibitory effect. These inhibitory effects of fixed cell sheets correlated well with both the amount of heparan sulphate relative to the total GAGs on the surface and to the saturation density of cell growth at each passage. These findings strongly suggest that heparan sulphate, or its complex, on the cell surface is involved in the regulation of cell proliferation.

Examination of ^{3}H-glucosamine incorporation into the cell-surface GAGs of skin fibroblasts from donors of different ages and from WS patients showed that heparan sulfate accumulates on the cell surface more rapidly in the WS cells and that more hyaluronic acid was excreted from WS cells into the medium. By using the fixed cell sheet assay, WS fibroblasts were confirmed to have a greater inhibitory effect on cell growth than age-matched normal fibroblasts. Thus, the role of cell-surface changes in the regulation of cell proliferation in normal aging and in the Werner syndrome should be given greater emphasis.

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