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Diminished Response of Werner's Syndrome Fibroblasts to Growth Factors PDGF and FGF

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Patients with Werner's syndrome, an autosomal recessive disorder, undergo an accelerated aging process that leads to premature death. Fibroblasts from such patients typically grow poorly in culture. Here it is shown that fibroblasts from a patient with Werner's syndrome have a markedly attenuated mitogenic response to platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). In contrast, they have a full mitogenic response to fetal bovine serum. Both PDGF binding and receptor numbers per cell are unaltered. The Werner's syndrome cells express high constitutive levels of collagenase in vitro. Although PDGF enhances collagenase expression through increased levels of hybridizable collagenase messenger RNA in normal skin fibroblasts, no induction of collagenase occurs in the Werner's syndrome fibroblasts. Moreover, the failure to respond to this agonist effect of PDGF is not restored by fetal bovine serum. The data suggest that failure of one or more PDGF-mediated pathways in Werner's syndrome cells may contribute to the phenotypic expression of the disorder.

WERNER'S SYNDROME IS AN AUTOsomal recessive disorder that is generally characterized by an apparent acceleration of many of the processes associated with aging. Some of the principal features, as defined by Thannhauser (*1*), are short stature with thin extremities and stocky trunk, premature graying of hair, premature baldness, patches of stiffened skin (especially in the face and lower extremities), trophic ulcers of the legs, juvenile cataracts, hypogonadism, tendency to diabetes, calcification of the blood vessels, osteoporosis, metastatic calcifications, and a tendency to occur in siblings (*1*). Growth of skin fibroblasts from Werner's syndrome patients in vitro is more difficult than that of normal skin fibroblasts (*1*). Furthermore, Werner's syndrome fibroblasts have a reduced life span in vitro and lengthened mean population doubling time (*2*). These observations, coupled with biochemical evidence for disordered connective tissue metabolism (*3*), suggest that a substantial component of the Werner's syndrome phenotype is manifested in mesenchymal tissues.

The mechanism underlying Werner's syndrome has not been identified, but the apparent premature aging has suggested that abnormal cellular growth and differentiation may be important in the phenotypic expression of Werner's syndrome. A number of peptide growth factors have been identified as being capable of regulating cellular growth and differentiation. The platelet-derived growth factor (PDGF) may be important in these processes, because in vitro it initiates cell proliferation, cell migration, and cellular activation associated with seemingly diverse processes such as inflammation, wound healing, and perhaps athero genesis (*4*), and possibly—by homology to the putative transforming protein of the simian sarcoma virus, $p28^{v\text{-}sis}$—in the molecular events that govern malignant transformation (*5*).

Skin fibroblasts synthesize collagenase as one of their principal gene products (*6*); this enzyme functions at the rate-limiting step in initiating collagen degradation and remodeling in a wide variety of morphogenic events, including wound healing (*7*). Because of the profound effects of PDGF on mesenchymal cells, we postulated that this protein also might influence certain biochemical events in fibroblasts essential to remodeling and wound healing. It was thus of interest to observe that PDGF stimulates the synthesis and secretion of collagenase by normal human skin fibroblasts under conditions in which no apparent increase in DNA synthesis is observed (*8*). Here, we used skin fibroblasts cultured from a patient with Werner's syndrome and from the offspring of a patient with Werner's syndrome (obligate heterozygote) to address several questions: (i) Are two agonist functions of PDGF—mitogenesis and stimulation of collagenase synthesis—altered in Werner's syndrome? (ii) Are the putative alterations specific to PDGF? (iii) What mechanisms are involved?

Initially we examined the replicative capacity in vitro of fibroblasts from age- and sex-matched normal subjects and patients with Werner's syndrome (Human Genetic Mutant Cell Repository, AG780). The mean population doubling times of each were not significantly different when low passage ($\leq$12) cells were cultured in 10% fetal bovine serum (FBS). Further, saturation densities of normal and Werner's syndrome cultures were equal. Confluent, quiescent fibroblasts in culture from normal individuals and from patients with Werner's syndrome were next stimulated with 100 ng/ml of PDGF. The incorporation of [^{3}H]thymidine into DNA of PDGF-stimulated control cells was 480 to 760% of that in nonstimulated cells, whereas [^{3}H] incorporation into Werner's cells stimulated by PDGF was only 108 to 144% of that in unstimulated cells in repeated experiments (Table 1). In cells from a Werner's syndrome heterozygote, PDGF stimulated [^{3}H]thymidine incorporation 153% above control cells (Table 1). In contrast, the Werner's syndrome cells responded normally to the mitogenic stimulus of 20% FBS as did the Werner's syndrome heterozygote cells. Whole human serum (WHS; 20%, pooled samples from adults) was a less effective mitogen than 20% FBS in the cultures of Werner's syndrome cells, perhaps because PDGF is the principal mitogenic protein in human serum for cells of mesenchymal origin. This suggests that the Werner's cells cultured in FBS may be responding to fetal growth-promoting activities that, as yet, are uncharacterized.

The difference in responsiveness of Werner's syndrome fibroblasts and normal control fibroblasts is not due to observable

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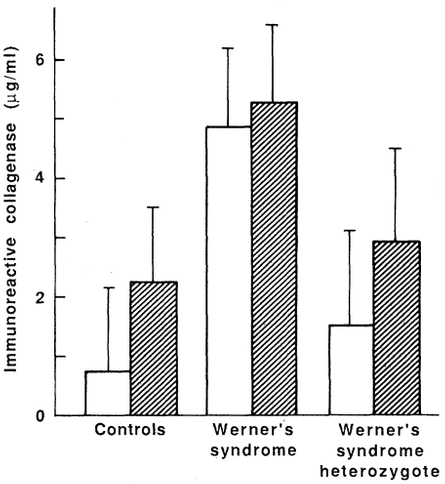


Fig. 1. Effect of PDGF on collagenase expression in cultures of Werner's syndrome fibroblasts. Cells were grown as described for Table 1 and PDGF was added as noted. Immunoreactive collagenase concentrations were determined as described (*8*). Data are presented as mean (±SE, $n = 9$) collagenase concentration in the absence (open bar) or presence (crosshatched bar) of 100 ng/ml of PDGF. Constitutive levels of collagenase in the Werner's syndrome cultures were sixfold (±1.5) greater than in control cultures ($P < 0.005$), threefold greater than in the heterozygote cultures ($P < 0.025$), and the approximately twofold (±1.8-fold) increase in collagenase in heterozygote was not significantly different from controls when analyzed by a computer-based t test. PDGF (100 ng/ml) stimulated collagenase about threefold in control cultures ($P < 0.025$), and 1.1-fold in cultures of Werner's cells (not significant).

differences in the number and apparent affinity of PDGF receptors (*9*). We found that the binding of ^{125}I-labeled PDGF was specific to both normal and Werner's syndrome cells and was reversible; the apparent dissociation constant (K_d) for the binding of PDGF to normal fibroblasts was $4.2 \pm 0.9 \times 10^{-9} M$ with $1.74 \pm 0.28 \times 10^5$ receptors per cell (mean ± SE), and for the Werner's syndrome cells the apparent K_d was $2.0 \pm 0.4 \times 10^{-9} M$ with $1.41 \pm 0.17 \times 10^5$ receptors per cell. The loss of responsiveness to PDGF in Werner's syndrome cells thus appears to be localized in pathways beyond the cell surface binding of PDGF.

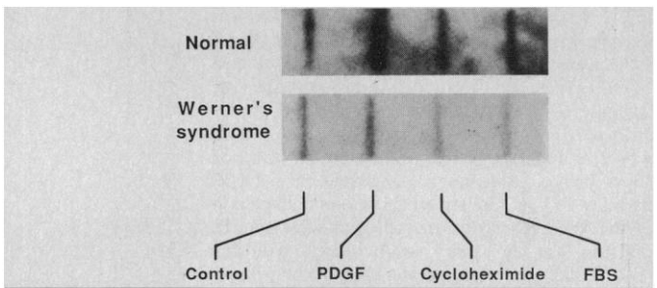
The results prompted us to look at the mitogenic response of cells from the Werner's syndrome patient and from the obligate heterozygote to other growth factors. To maximize any differences in response, we used less-than-saturating concentrations of the various growth factors (Table 2). The responsiveness of Werner's cells to PDGF was again markedly attenuated, as was their response to 20% WHS. In contrast, at 2.5 ng/ml of epidermal growth factor, the stimulation of [^{3}H]thymidine incorporation in the Werner's cells was enhanced about 1.7 to 1.8 times and was thus only slightly blunted compared to the control cells. At 25 ng/ml, fibroblast growth factor elicited effectively no increase in [^{3}H]thymidine incorporation into the DNA of Werner's syndrome cells. Intermediate results were observed with obligate heterozygote cells in response to fibroblast growth factor. Thus, the blunted mitogenic response of fibroblasts from Werner's syndrome patients is not limited to PDGF; nevertheless, the striking response of these cells to FBS establishes their potential for a full mitogenic response and suggests the existence of different pathways eliciting mitogenic responses to different growth factors.

Since PDGF stimulates the synthesis and secretion of collagenase by normal cells (*8*), we also examined whether this function was abrogated in the Werner's syndrome cells. Culturing normal skin fibroblasts in the presence of increasing concentrations of PDGF resulted in a dose-dependent increase in immunoreactive collagenase in the culture medium to levels about three times over control values. Half-maximal secretion occurred at 30 to 40 ng/ml of PDGF. In contrast, the stimulation of collagenase synthesis and secretion in the cultures of Werner's syndrome fibroblasts was markedly blunted (~1.3 times). An intermediate (~1.8-fold) stimulation of collagenase synthesis was observed in the heterozygote cells. In each case, maximum stimulation was achieved at 90 to 125 ng/ml of PDGF. The use of higher concentrations of PDGF (up to 200 ng/ml) did not result in further augmentation of collagenase synthesis in any of the cell strains examined. The influence of PDGF on a large series of control cells and cells from the Werner's syndrome patient and Werner's syndrome heterozygote was next examined (Fig. 1). The constitutive levels of collagenase in the cultures of Werner's syndrome cells were about sixfold greater than in control cultures and about threefold greater than in the cultures of heterozygote cells. Although constitutive levels of collagenase in the cultures of Werner's syndrome heterozygote cells were intermediate between controls and the homozygous mutant cells, the approximate twofold increase in collagenase was not significantly different from controls. The addition of 100 ng/ml of PDGF to the cultures stimulated the synthesis and secretion of collagenase by about three times in control cultures, about two times in the heterozygote cultures, but only 1.1 times in the cultures of Werner's syndrome cells.

Our previous studies in normal cells established that the PDGF-mediated increased synthesis and secretion of collagenase was associated with increased levels of translatable collagenase messenger RNA (mRNA) (*8*). As shown in Fig. 2, the increase in collagenase synthesis elicited by PDGF in control cells was paralleled by an increase in hybridizable collagenase mRNA. The Werner's syndrome cells appeared to express

Table 1. Responsiveness of fibroblasts from control subjects and patients with Werner's syndrome to PDGF and fetal bovine serum (FBS). The fibroblasts were used at low passage and maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS with penicillin and streptomycin. Cultures were expanded by trypsinization and subsequent culture after a 1:3 splitting. Mitogenic activity was measured by the incorporation of [^{3}H]thymidine into trichloroacetic acid (TCA)–precipitable material. Confluent fibroblasts in 48-well plates were "serum starved" for 48 hours in DMEM medium with 0.2% FBS and then cultured in DMEM with 5% plasma-derived serum. [^{3}H]Thymidine (10 μCi/ml; 79.4 Ci/mM, New England Nuclear) was added together with PDGF, FBS, or whole human serum (WHS) for 41 to 48 hours. Cells were washed with phosphate-buffered saline at *p*H 7.4 and fixed with 10% TCA before the incorporated radioactivity was measured. Control [^{3}H]thymidine incorporation was 833 ± 188 dpm for normal cells, 568 ± 111 dpm for Werner's cells, and 718 ± 9 dpm for Werner's heterozygote cells. The data represent the mean ± SE of four or more cultures except in the case of experiment 3 in which two cultures each were incubated with 20% FBS. ND, assay not done.

Culture	[^{3}H]Thymidine incorporation (% of control)		
	Normal	WS	WS heterozygote
	Experiment 1		
PDGF (100 ng/ml)	640 ± 4	144 ± 10	ND
FBS (20%)	240 ± 8	341 ± 8	ND
WHS (20%)	674 ± 2	153 ± 1	ND
	Experiment 2		
PDGF (100 ng/ml)	760 ± 10	137 ± 6	ND
FBS (20%)	238 ± 22	397 ± 13	ND
WHS (20%)	570 ± 6	108 ± 8	ND
	Experiment 3		
PDGF (100 ng/ml)	482 ± 213	108 ± 2	153 ± 14
FBS (20%)	413 ± 2	347 ± 10	841 ± 20



Culture		(Relative hybridization)		
Normal	1.0	3.4±0.9	1.1±0.2	3.9±0.5
Werner's syndrome	1.0	1.3±0.2	1.0±0.1	0.9±0

Fig. 2. Steady-state levels of collagenase mRNA in Werner's syndrome fibroblasts. Cells were seeded at a density of 50,000 cells per well in Costar six-well plates, grown to confluence and serum starved for 48 hours in DMEM + 0.2% fetal bovine serum (FBS). Cells were then maintained for 24 hours in DMEM in the presence or absence of PDGF (100 ng/ml), cycloheximide (10 μg/ml), or 10% FBS. Medium was removed for immunoassay of collagenase and cells were washed with phosphate-buffered saline and removed by trypsinization and counted. Cytoplasmic RNA was prepared by the NP-40–formaldehyde method (*21*) and volumes were adjusted so that hybridizable RNA was reflected on a "per cell" basis. RNA was applied to nitrocellulose filters with the use of a Schleicher & Schuell slot blot template and hybridized with a ^{32}P-labeled nick-translated complementary DNA clone for human skin collagenase as described (*22*). Relative hybridizations were determined by scanning the radiographs at 600 n*M* and determining the area under each peak and were calculated on a per cell basis. The data are presented as mean (±SE) hybridization to mRNA from the stimulated cultures compared to hybridizable mRNA in control cultures containing no agonist in three separate experiments. Because of the high constitutive steady-state levels of collagenase mRNA, the filter from the Werner's syndrome cells contained one-eighth as much RNA as the normal control filter. This dilution was used for hybridization so that scanning of the film remained in the linear range of assay for both normal and Werner's cells. Immunoreactive collagenase measured in the culture medium of normal cells was 321 ± 42% of control for PDGF cultures, 78 ± 18% control for cycloheximide cultures, and 249 ± 51% of control for FBS cultures. In the Werner's syndrome cultures, immunoreactive collagenase was 115 ± 16% of control in the PDGF cultures, 92 ± 16% of control in the cycloheximide cultures, and 97 ± 17% of control in the FBS cultures.

high constitutive levels of collagenase mRNA but did not express greater mRNA levels in response to PDGF; these constitutive levels were sufficiently high that the mRNA used for hybridization was diluted 1:8 in order to provide comparable levels for analysis with mRNA from control cells and to ensure that the levels measured were within the linear range of assay for mRNA of both normal and Werner's cells. It is interesting that the addition of 10% FBS enhanced steady-state levels of collagenase mRNA in the normal cells by about three to four times but did not alter the expression of collagenase mRNA in the Werner's syndrome cells. The mRNA levels measured (Fig. 2) were observed in repeated experiments and in each instance were applied for analysis in direct proportion to cell number.

These data indicate that the failure of the Werner's syndrome cells to respond to PDGF is not unique to this growth factor and is probably not a defect specific to the PDGF receptor protein. Other activities initiated after exposure of cells to growth factors include phosphoinositide turnover (*10, 11*), the activation of protein kinase C (*11, 12*), the early induction of the *fos* and *myc* oncogenes (*13–19*), and an increase in cellular levels of protein phosphotyrosine (*14, 20*). It seems significant that the Werner's syndrome cells are able to respond normally to the mitogenic effects of FBS, since this establishes that the Werner's syndrome cells can mount a full mitogenic response equal to, or in some cases greater than (Table 1), that of normal fibroblasts. That Werner's syndrome cells do not respond to serum from adults further suggests that a growth-promoting activity associated with fetal serum is not present in serum from adults. Whether the putative defect that is restricted to mitogenic responsiveness to adult serum contributes to the premature aging of Werner's patients cannot be established by the data.

A second interesting aspect of these results is the high constitutive expression of collagenase and the failure of the Werner's cells to respond to PDGF by inducing additional collagenase expression. It seems plausible that the secreted collagenase, possibly resulting in increased turnover of skin collagen and connective tissue remodeling, may play a role in the cutaneous atrophy and ulcerations that are phenotypic characteristics of the premature aging. It is also possible that the increased expression of collagenase may be important in understanding other abnormalities expressed by patients with Werner's syndrome, such as the increased incidence of neoplasia (*1*). Whether these differences from normal cells are related to the diminished mitogenic response seen in Werner's cells is not clear, although it is noteworthy that, in contrast to the corrective effect on the mitogenic response, fetal bovine serum did not enhance in vitro expression of collagenase or induce collagenase mRNA (Fig. 2). This indirectly suggests the possible existence in the normal situation of two PDGF-mediated pathways: one that leads to mitogenesis and can be corrected by FBS in Werner's syndrome fibroblasts, and another that results in enhanced collagenase expression but is not corrected by FBS in Werner's syndrome fibroblasts. Thus, from a broader biologic perspective, these mutant fibroblasts appear to represent an important experiment of nature that will be of value in defining separate pathways activated during PDGF-evoked responses and in elucidating the nature of additional growth-promoting agents in fetal serum.

Table 2. Responsiveness of control and Werner's syndrome fibroblasts to various growth factors. Cells were cultured as noted for Table 1. The PDGF and WHS were obtained as previously described (*9*); the epidermal growth factor (EGF) and fibroblast growth factor (FGF) were obtained from Collaborative Research. [^{3}H]Thymidine incorporation assays were performed as noted for Table 1, except that cells were cultured in 12-well plates, and results are expressed as the percentage of incorporation seen in control cultures containing no growth factors. Control [^{3}H]thymidine incorporation into the normal cells was 2927 dpm, into Werner's cells was 2330 dpm, and into the Werner's cell heterozygotes was 1398 dpm. The data represent the mean ± SE of four or more cultures.

Culture	[^{3}H]Thymidine incorporation (% of control)		
	Normal	WS	WS heterozygote
PDGF (25 ng/ml)	198 ± 29	116 ± 9	125 ± 10
WHS (20%)	674 ± 2	153 ± 1	ND
EGF (2.5 ng/ml)	317 ± 87	174 ± 15	171 ± 11
FGF (25 ng/ml)	303 ± 154	110 ± 1	186 ± 3

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A Selective Imidazobenzodiazepine Antagonist of Ethanol in the Rat

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Ethanol, at pharmacologically relevant concentrations of 20 to 100 m*M*, stimulates γ-aminobutyric (GABA) receptor–mediated uptake of ^{36}Cl-labeled chlorine into isolated brain vesicles. One drug that acts at GABA-benzodiazepine receptors, the imidazobenzodiazepine Ro15-4513, has been found to be a potent antagonist of ethanol-stimulated $^{36}Cl^-$ uptake into brain vesicles, but it fails to antagonize either pentobarbital- or muscimol-stimulated $^{36}Cl^-$ uptake. Pretreatment of rats with Ro15-4513 blocks the anticonflict activity of low doses of ethanol (but not pentobarbital) as well as the behavioral intoxication observed with higher doses of ethanol. The effects of Ro15-4513 in antagonizing ethanol-stimulated $^{36}Cl^-$ uptake and behavior are completely blocked by benzodiazepine receptor antagonists. However, other benzodiazepine receptor inverse agonists fail to antagonize the actions of ethanol in vitro or in vivo, suggesting a novel interaction of Ro15-4513 with the GABA receptor–coupled chloride ion channel complex. The identification of a selective benzodiazepine antagonist of ethanol-stimulated $^{36}Cl^-$ uptake in vitro that blocks the anxiolytic and intoxicating actions of ethanol suggests that many of the neuropharmacologic actions of ethanol may be mediated via central GABA receptors.

ETHANOL IS ONE OF MAN'S OLDEST and most ubiquitous psychoactive drugs. The acute administration of relatively low doses of ethanol results in anticonflict and anxiolytic activity in laboratory animals (*1*) and man (*2*), and higher doses produce sedation, intoxication, coma, and death (*3*). Although ethanol produces a variety of diverse central nervous system (CNS) effects (*4*), it is still unclear which, if any, of these actions are related to its behavioral effects. Moreover, it is generally accepted that the pharmacological actions of ethanol and related short-chain alcohols result from relatively nonspecific interactions with biomembranes (*5*) because many of the behavioral effects of short-chain alcohols are highly correlated with both their lipid solubilities (*6*) and membrane fluidizing and disordering properties (*7*). However, these nonspecific membrane effects of alcohol may result in specific alterations in membrane function, including changes in the conformation of membrane-bound receptors such as those coupled to adenylate cyclase (*8*) and ion channels (*9*). We have reported that, at pharmacologically relevant concentrations (20 to 100 m*M*), ethanol and related short-chain alcohols stimulate γ-aminobutyric acid (GABA) receptor–mediated uptake of ^{36}Cl-labeled chlorine into isolated brain vesicles, and that this effect was correlated with both their membrane-buffer partition coefficients and intoxication potencies in rats (*10*). The action of short-chain alcohols in stimulating $^{36}Cl^-$ uptake in vitro appears to be mediated via the GABA-coupled Cl^- ion channel because the effects are blocked by the specific $GABA_A$ receptor antagonist bicuculline and the Cl^- channel antagonist picrotoxin (*10*).

In view of the many common neuropharmacological actions of benzodiazepines, barbiturates, and ethanol (*1, 3*) and, because both benzodiazepines and barbiturates have been shown to augment GABAergic neurotransmission (*11*), we have postulated that ethanol's action at the level of the GABA-coupled Cl^- ion channel may underlie many of its behavioral properties (*10*). In the course of studying the interactions of benzodiazepines, barbiturates, and ethanol on GABA receptor–mediated $^{36}Cl^-$ uptake in vitro, we found that the imidazobenzodiazepine Ro15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5α][1,4] benzodiazepine-3-carboxylate) (*12*) potently and selectively antagonized the ability of ethanol (but not pentobarbital or muscimol) to stimulate $^{36}Cl^-$ uptake in vitro. Moreover, parenteral administration of Ro15-4513 to rats resulted in a blockade of the anticonflict and intoxicating actions of ethanol. Both the in vitro and in vivo effects of Ro15-4513 in antagonizing the actions of ethanol were prevented by the central benzodiazepine receptor antagonists Ro15-1788 and CGS-8216, suggesting a novel interaction of Ro15-4513 with the benzodiazepine receptor complex. Thus, many of the behavioral and biochemical actions of ethanol may be mediated by central GABA-benzodiazepine receptors.

In our initial experiments, we examined a

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