

ORIGINAL ARTICLE

Age related expression of Werner's syndrome protein in selected tissues and coexpression of transcription factors

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Aims: Werner's syndrome (WS) is an uncommon autosomal recessive disease resulting from mutational inactivation of human WRN helicase, Werner's syndrome protein (WRNp). Patients with WS progressively develop a variety of aging characteristics after puberty. The aim of this study was to determine the distribution of WRNp and the expression of the transcription factors regulating WRN gene expression in a variety of human organs in an attempt to understand the WS phenotype.

Methods: Tissue specimens were obtained from 16 controls aged from 27 gestational weeks to 70 years of age and a 56 year old female patient with WS. The distribution of WRNp and the expression of the transcription factors regulating WRN gene expression—SP1, AP2, and retinoblastoma protein (Rb)— were studied in the various human organs by immunohistochemical and immunoblot analyses.

Results: In the healthy controls after puberty, high expression of WRNp was detected in seminiferous epithelial cells and Leydig cells in the testis, glandular acini in the pancreas, and the zona fasciculata and zona reticularis in the adrenal cortex. In addition, the SP1 and AP2 transcription factors, which regulate WRNp gene expression, appeared in an age dependent manner in those regions where WRNp was expressed. In controls after puberty, SP1 was expressed in the testis and adrenal gland, whereas AP2 was expressed in the pancreas.

Conclusions: These findings suggest that the age specific onset of WS may be related to age dependent expression of WRNp in specific organs.

Werner's syndrome (WS) is an uncommon autosomal recessive disease resulting from mutational inactivation of the human WRN helicase gene, which encodes both helicase and exonuclease activities, and is found at 8p11-12.^{1,2} WRN helicase is one of five human RecQ helicases, and is one of the three such genes that have been related to a heritable human disease; namely: Bloom syndrome (BLM)³ and Rothmund-Thomson syndrome (RTS).⁴

The characteristic phenotype of WS is premature aging. After puberty, affected individuals rapidly develop greying and loss of hair, scleroderma-like skin changes, osteoporosis, atherosclerosis, non-insulin dependent diabetes mellitus, and hypogonadism; these patients also have an increased risk of cancer.⁵ At the cellular level, cultured cells from patients with WS have a shortened life span and prolonged S phase of the cell cycle.⁶ In addition, increased mutation frequency, chromosomal instability, and rapid senescence of fibroblast cultures are seen in cells from patients with WS.⁷⁻⁹

"The characteristic phenotype of WS is premature aging"

The 5' upstream region of the WRN gene contains three cis regulating elements: an SP1 element, an AP2 element, and a retinoblastoma (Rb) control element (RCE). WRN expression is regulated mainly by the SP1 transcriptional control system.¹⁰ Despite this molecular information we do not understand why the WS phenotype is only expressed after puberty. The hypotheses are that: (1) WRN is expressed after puberty in specific tissues, and/or (2) the transcription factors regulating WRN gene expression are manifested after puberty in those tissues that express WRN. Thus, in our present study, using immunohistochemical and immunoblot analyses of various postmortem human tissues we investigated the

expression of WS protein (WRNp) and transcription factors, SP1, AP2, and Rb in an attempt to clarify the pathogenetic importance of WRN.

MATERIALS AND METHODS

Postmortem tissues

Sets of postmortem tissue specimens (brain, liver, pancreas, testis, ovary, kidney, adrenal gland, heart, spleen, and intestine) were obtained from 16 controls and a female patient with WS. The ages of the controls ranged from 27 gestational weeks to 70 years and the patient with WS was 56 years old.¹¹ In these cases, informed consent was obtained in writing. The necropsies were performed within 24 hours of death, and usually within 12 hours.

Immunohistochemistry

The sections for immunohistochemistry were dewaxed and pretreated with 0.3% hydrogen peroxide in methanol for 15 minutes to abolish endogenous peroxidase activity, and then incubated for 30 minutes in the presence of 10% normal rabbit or goat serum to block non-specific binding. The sections were then incubated with a mouse monoclonal antibody, 8H3, which is specific to the N-terminus of human WRNp (diluted 1/500),¹² a goat polyclonal antibody against human SP1 (diluted 1/10) (PEP2-G; Santa Cruz Biotechnology, Santa Cruz, California, USA), or a rabbit polyclonal antibody against human AP2 (diluted 1/500) (C-18, Santa Cruz Biotechnology) overnight at 4°C. This was followed by incubation with biotinylated rabbit antimouse IgG/A/M, goat antirabbit IgG, or

Abbreviations: BLM, Bloom syndrome; DHEA-S, dehydroepiandrosterone sulphate; Rb, retinoblastoma protein; RCE, retinoblastoma control element; RTS, Rothmund-Thomson syndrome; WS, Werner's syndrome; WRNp, Werner's syndrome protein

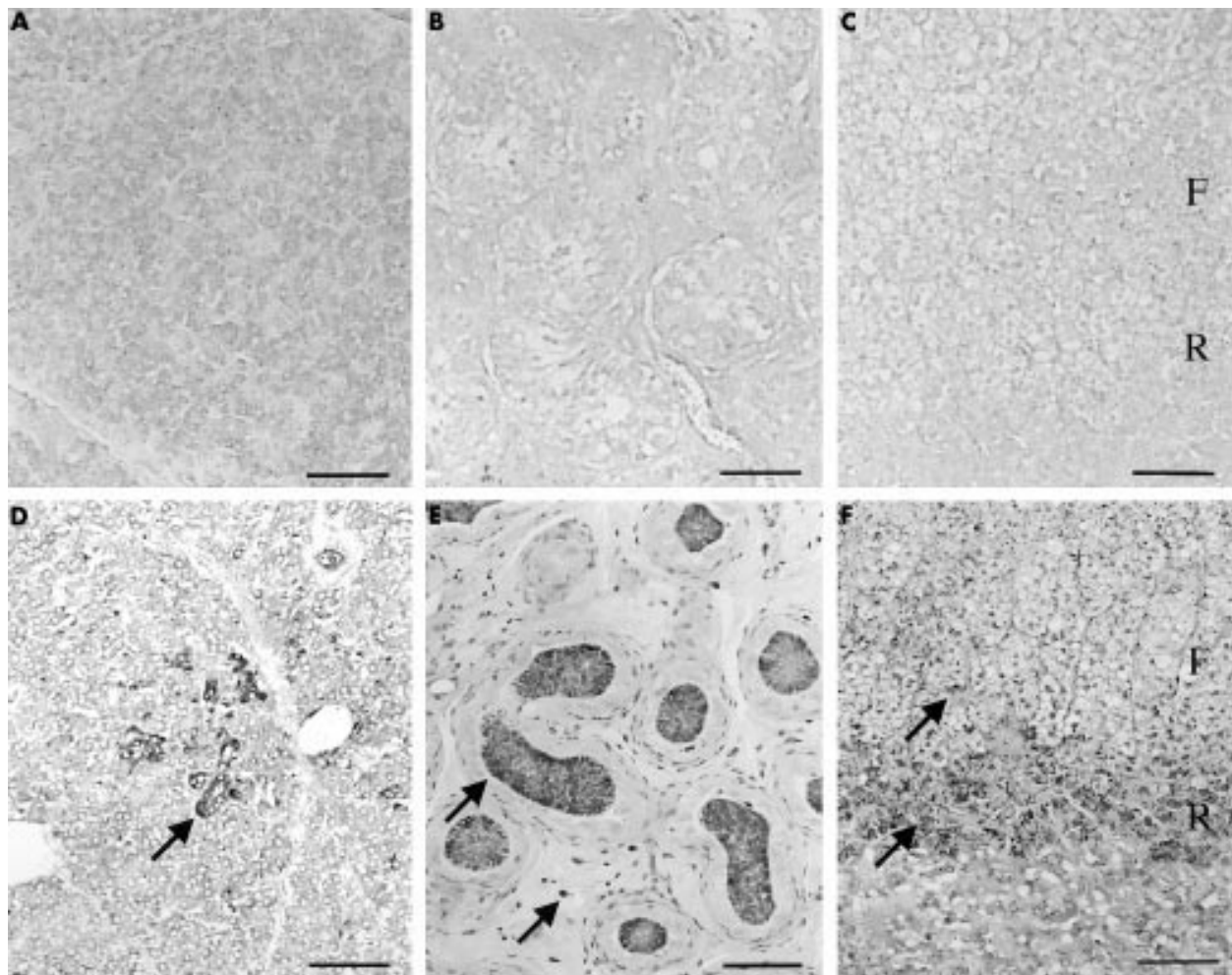


Figure 1 Immunoperoxidase staining of the organs, showing age dependent changes in Werner's protein (WRNp) immunoreactivity. Pancreas at (A) 11 years and (D) 32 years; seminiferous tubules in the testis at (B) 11 years and (E) 32 years; cortex of the adrenal gland at (C) 11 years and (F) 32 years. Arrows indicate the WRNp positive cells. F, zona fasciculata; R, zona reticularis. Scale bar, 1 μ m.

rabbit anti-goat IgG (Nichirei, Tokyo, Japan), respectively, for one hour at room temperature and then incubation with peroxidase conjugated streptavidin (Nichirei) for 30 minutes at room temperature. After using the TSA Plus kit (NEN Life Products, Boston, Massachusetts, USA), the immunoproducts were visualised by the addition of 0.02M diaminobenzidine tetrahydrochloride in 0.05M Tris buffer, pH 7.6, containing 0.006% hydrogen peroxide. Nuclei were counterstained with haematoxylin. The detailed procedures for preparing the monoclonal antibody 8H3, and its biochemical and immunocytochemical characterisation, have been published elsewhere.^{12, 13} In addition, all specimens were stained with haematoxylin and eosin as a routine examination.

Immunoblot analysis

Frozen sections of frontal lobes, pancreas, and kidney from the controls aged 27 gestational weeks, 11 years, and 64 years were used for nuclear and cytoplasmic protein extraction. The samples were thawed and homogenised in the buffer from the nuclear and cytoplasmic extraction reagents kit (Pierce, Rockford, Illinois, USA), according to the manufacturer's instructions. After the protein concentration was measured, the samples were incubated for two minutes at 95°C. The protein samples were separated by 7.5% polyacrylamide gel electrophoresis and then transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Massachusetts, USA). The membrane was incubated overnight at 4°C with primary antibody against human WRNp

diluted 1/1000, followed by antimouse IgG and horseradish peroxidase labelled whole antibody diluted 1/5000 (Amersham Pharmacia Biotech, Amersham, Buckinghamshire, UK). The colour was developed with the aid of an ECL kit (Amersham Pharmacia Biotech).

RESULTS

Immunohistochemistry to detect WRNp and relevant transcription factors in human tissues

We examined the expression of WRNp in brain, liver, pancreas, testis, ovary, kidney, adrenal gland, heart, spleen, and intestine obtained from controls of various ages by immunohistochemical analyses. Immunoreactivity for WRNp was not detected in the organs from the controls during the neonatal period and childhood (fig 1A, B, C). Appreciable amounts of WRNp first appeared in selective tissues—such as glandular acini of the pancreas, seminiferous epithelial cells and Leydig cells in the testis, and the zona fasciculata and zona reticularis in the adrenal cortex—from the age of 17 in controls, and was detected persistently after adolescence (fig 1D, E, F). However, immunoreactivity for WRNp was not detected in the other organs at all ages. WRNp was not detected in these organs from the 56 year old female patient with WS (data not shown).

Next, we studied the expression of the transcription factors regulating WRN gene expression in the pancreas, adrenal gland, and testis by means of immunostaining. SP1 and AP2 could not be detected in the tissues from young controls (fig

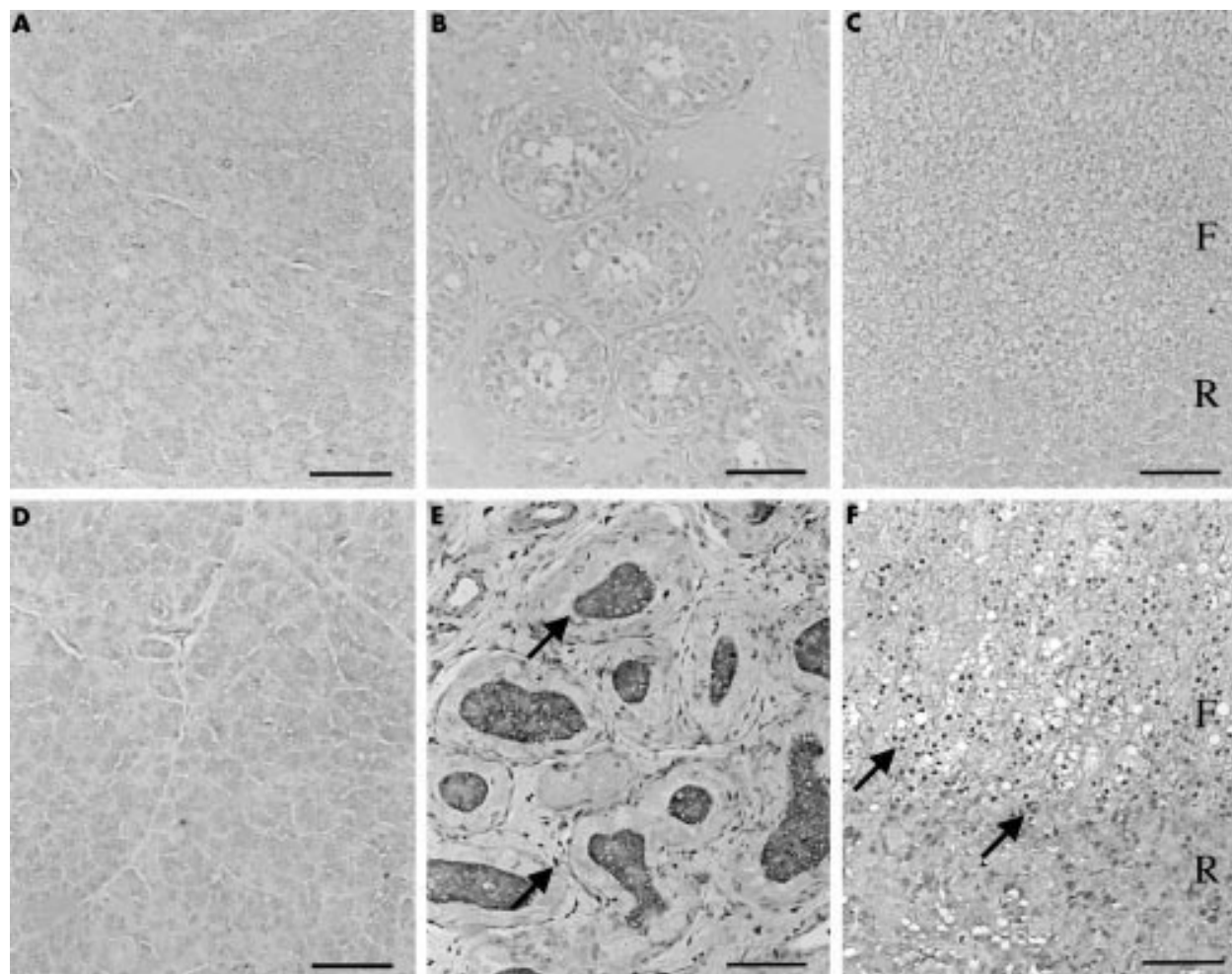


Figure 2 Immunoperoxidase staining of the organs, showing age dependent changes in SP1 immunoreactivity. Pancreas at (A) 11 years and (D) 32 years; seminiferous tubules in the testis at (B) 11 years and (E) 32 years; cortex of the adrenal gland at (C) 11 years and (F) 32 years. Arrows indicate SP1 positive cells. F, zona fasciculata; R, zona reticularis. Scale bar, 1 μ m.

2A, B, C and 3A, B, C), whereas SP1 was seen in seminiferous epithelial cells and Leydig cells in the testis (fig 2E) and the zona fasciculata and zona reticularis in the adrenal cortex (fig 2F), but not in the glandular acini of the pancreas (fig 2D) from controls after puberty. Immunoreactivity for AP2 was detected in the glandular acini of the pancreas (fig 3D), but not in the seminiferous epithelial cells and Leydig cells in the testis (fig 3E), or in the zona fasciculata and zona reticularis in the adrenal gland (fig 3F) from controls after puberty. In contrast, immunoreactivity for the Rb protein was not seen in the pancreas, adrenal cortex, or testis at all stages (data not shown).

Immunoblot analysis for WRNp in tissues from young and old healthy subjects

Immunoblot analysis showed that WRNp was abundant in the cytosol fraction of the pancreas from a 64 year old control (fig 4; lane 8). In contrast, bands were not detected in the cytosol fraction from young controls (fig 4; lanes 1–6) and in the frontal lobe and the kidney from the 64 year old control (fig 4; lanes 7, 9). Surprisingly, the WRNp specific bands were not detected in the nuclear fraction from all controls when 50 μ g of nuclear protein was used (data not shown).

DISCUSSION

In our study, we first demonstrated the age related expression of WRN in selected tissues and the simultaneous expression of some transcription factors involved in the upregulation of the

WRN gene. High expression of WRNp after puberty was seen in the seminiferous epithelial cells and Leydig cells in the testis, glandular acini in the pancreas, and the zona fasciculata and zona reticularis of the adrenal cortex in controls. Immunoblot analyses also showed that WRNp appeared in these tissues after puberty. We found that WRNp was abundant in the cytosol obtained from the frozen specimens; this expression pattern implies that WRNp has a dual nuclear and cytoplasmic localisation in the cell. It has been reported that p53 is stored in the cytoplasm, closely associated with the cytoskeletal actin filaments, and that some of this p53 moves into the nucleus to initiate gene activation in resting fibroblasts.¹⁴ On the basis of this evidence, we speculate that WRNp is stored in the cytoplasm and some of the WRNp moves into the nucleus for DNA repair. The expression profiles of the five human RecQ helicases differ. As measured by northern blot analysis, the WRN gene is expressed highly in the pancreas and the testis, whereas the BLM gene is expressed predominantly in the thymus and the testis, like the RTS helicase gene. The RecQL gene appears to be expressed ubiquitously, like RecQ5.¹⁵ The present immunological results with WRNp are in good agreement with those previously published by Kitao *et al* using northern blot analysis.¹⁵

“We speculate that Werner’s syndrome protein is stored in the cytoplasm and some of this protein moves into the nucleus for DNA repair”

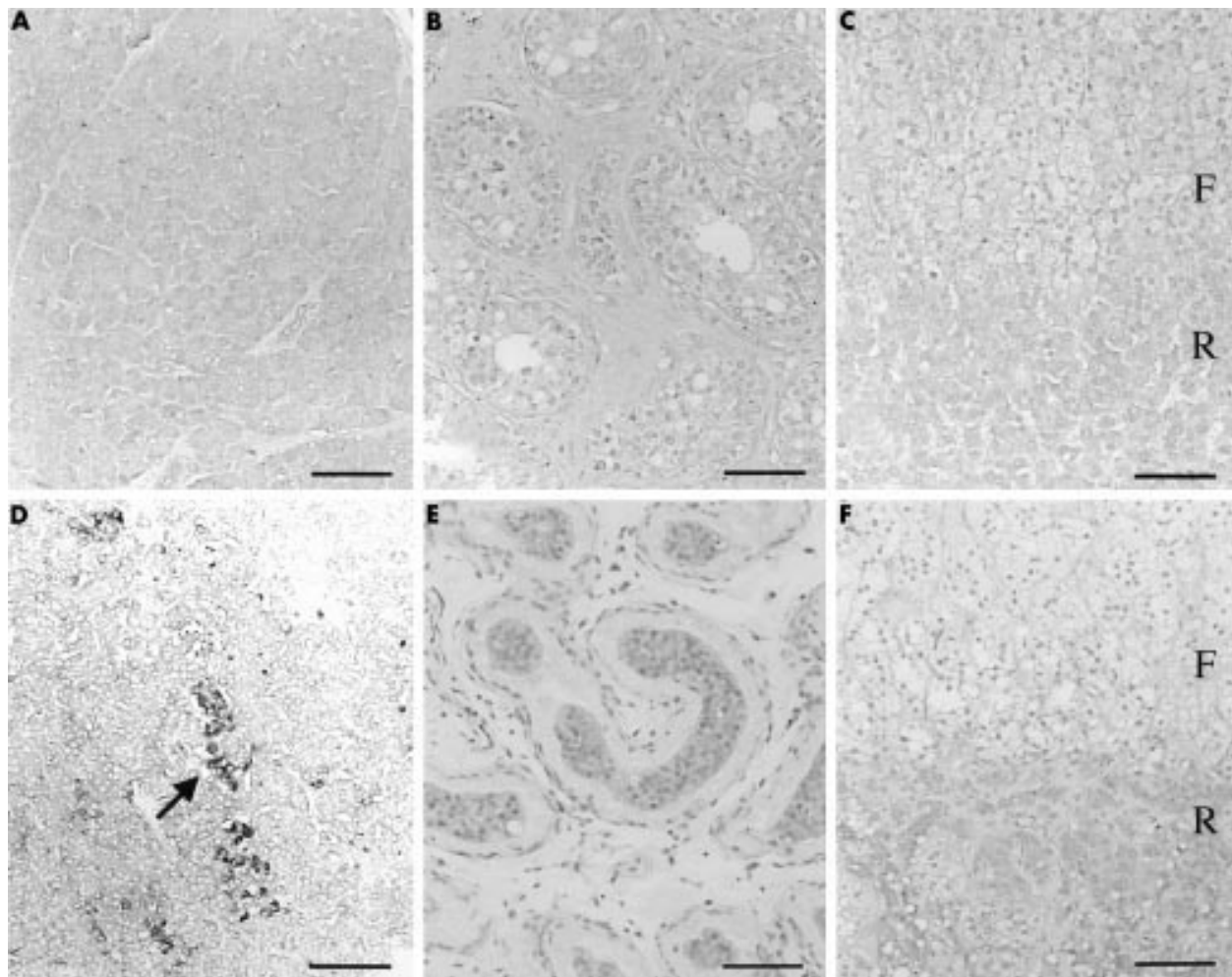


Figure 3 Immunoperoxidase staining of the organs, showing age dependent changes in AP2 immunoreactivity. Pancreas at (A) 11 years and (D) 32 years; seminiferous tubules in the testis at (B) 11 years and (E) 32 years; cortex of the adrenal gland at (C) 11 years and (F) 32 years. Arrows indicate AP2 positive cells. F, zona fasciculata; R, zona reticularis. Scale bar, 1 µm.

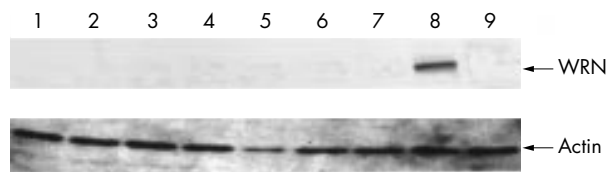


Figure 4 Immunoblot of cytosol extracts probed with the anti-WRNp antibody, showing age dependent changes in Werner's protein (WRNp) immunoreactivity. Lanes 1–3, tissues from the control subject at 27 gestational weeks: frontal lobe (lane 1), pancreas (lane 2), kidney (lane 3); lanes 4–6, tissues from the 11 year old control subject: frontal lobe (lane 4), pancreas (lane 5), kidney (lane 6); lanes 7–9, tissues from the 64 year old control subject: frontal lobe (lane 7), pancreas (lane 8), kidney (lane 9). Antiserum demonstrated the presence of the 170 kDa WRNp in the pancreas from the 64 year old control subject. An aliquot of 50 µg of extracted protein was applied to each lane. The lower figure shows immunoblot analysis of actin as a control for loading.

We demonstrated that the SP1 and AP2 transcription factors, which regulate WRN gene expression, also appeared in an age dependent manner in the regions where WRNp was expressed. In the controls after puberty, SP1 was expressed in the seminiferous epithelial cells and Leydig cells of the testis, and in the zona fasciculata and zona reticularis of the adrenal cortex, whereas AP2 was expressed in the glandular acini of the pancreas. These findings suggest that the copy number of

these transcription factors is also increased after puberty, resulting in the upregulation of WRN gene expression.

It is noticeable that those organs that express high amounts of WRNp are also responsible for the secretion of sex hormones (androgen and testosterone). The sex hormonal disturbances seen in aging occur together with a decrease in the secretion of androgen and oestrogen in the testicles. An important decrease in adrenal androgen secretion has been noted in both sexes. These hormonal disturbances are thought to promote aging, especially in bone, muscle, skin, and mucous membranes.¹⁶ Testosterone, which is not only responsible for the development of male secondary sexual characteristics at puberty, but is also essential for the continued function of the seminiferous epithelium, is the principal hormone secreted by Leydig cells. The zona fasciculata and the zona reticularis are thought to be responsible for the secretion of androgens. In the patient with WS, the lack of WRN gene expression in the Leydig cells, zona fasciculata, and zona reticularis may cause an accelerated cellular senescence as a result of a reduction in androgen and testosterone secretion. In fact, in the patient with WS, microscopic examination revealed atrophy of the zona fasciculata and zona reticularis, which was more pronounced than that seen in the older control patients.

To determine whether there was a disturbance of sex hormonal secretions in the patient with WS, we analysed the concentration of dehydroepiandrosterone (DHEA) in blood from this patient when she was admitted to our hospital.

Take home messages

- The WRN gene was expressed in selected tissues after puberty together with the transcription factors SP1 and AP2
- In the patient with WS, microscopic examination revealed atrophy of the zona fasciculata and zona reticularis, which was more pronounced than that seen in the older control patients
- Those organs that express high amounts of Werner's syndrome protein (WRNp) are also responsible for the secretion of sex hormones (androgen and testosterone)
- The lack of WRNp functions in selected organs closely correlate with the Werner's syndrome phenotype

DHEA, which is a precursor of androgen and acts as androgen itself, is produced principally in the zona fasciculata and the zona reticularis of the adrenal cortex, and after sulphate conjugation (to produce DHEA-S), is secreted into blood. The concentration of DHEA-S (230 ng/ml) in our patient with WS was lower than that seen in age matched female controls (normal range, 400–3500 ng/ml).

Endocrine and metabolic abnormalities have been reported in patients with WS when compared with normal, aged subjects.¹⁷ The serum testosterone concentrations of patients with WS were lower than those of age matched controls, and testicular biopsy revealed more pronounced atrophy than that seen in aged subjects.¹⁷

In conclusion, the WRN gene was expressed in selected tissues after puberty, and the transcription factors were coexpressed. The lack of WRNp functions in selected organs closely correlate with the WS phenotype.

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