

Subcytotoxic H₂O₂ Stress Triggers a Release of Transforming Growth Factor- β 1, Which Induces Biomarkers of Cellular Senescence of Human Diploid Fibroblasts*

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Stress-induced premature senescence (SIPS) is induced 3 days after exposure of human diploid fibroblasts to subcytotoxic oxidative stress with H₂O₂, with appearance of several biomarkers of replicative senescence. In this work, we show that transforming growth factor- β 1 (TGF- β 1) regulates the induction of several of these biomarkers in SIPS: cellular morphology, senescence-associated β -galactosidase activity, increase in the steady-state level of fibronectin, apolipoprotein J, osteonectin, and SM22 mRNA. Indeed, the neutralization of TGF- β 1 or its receptor (TGF- β RII) using specific antibodies decreases sharply the percentage of cells positive for the senescent-associated β -galactosidase activity and displaying a senescent morphology. In the presence of each of these antibodies, the steady-state level of fibronectin, osteonectin, apolipoprotein J, and SM22 mRNA is no more increased at 72 h after stress. Results obtained on fibroblasts retrovirally transfected with the human papillomavirus E7 cDNA suggest that retinoblastoma protein (Rb) regulates the expression of TGF- β 1 in stressful conditions, leading to SIPS and overexpression of these four genes.

Normal human diploid fibroblasts (HDFs)¹ exposed to various types of noncytotoxic oxidative stress display a senescent-like phenotype coined “stress-induced premature senescence” or SIPS (1, 2). Such stressful conditions include exposure to hydrogen peroxide (3, 4), *tert*-butylhydroperoxide (*t*-BHP) (5), hyperoxia (6), UV light (7), and radioactivity (8). Many biomarkers of replicative senescence appear in SIPS: typical cell mor-

phology (5), irreversible growth arrest, lack of response to mitogenic stimuli (4), sharp decrease of the DNA synthesis, and an increase in cells positive for the senescent-associated β -galactosidase activity (SA β -gal) (9). A long term overexpression of the cyclin-dependent kinase inhibitor p21^{waf-1} was observed in SIPS induced by H₂O₂ (10) or *t*-BHP (9). p21^{waf-1} inhibits the cyclin D/cyclin-dependent kinase 4 and 6 complexes, leading to hypophosphorylation of the retinoblastoma protein (Rb). A long term hypophosphorylation of Rb over several weeks was indeed observed in SIPS induced by H₂O₂ or *t*-BHP, explaining the block of the cell cycle, through Rb-mediated inhibition of the E₂F transcription factor (9, 10). Last, several genes overexpressed in senescent HDFs, such as fibronectin, osteonectin, SM22, and apolipoprotein J (clusterin), displayed a similar increase in mRNA level in SIPS induced by *t*-BHP or H₂O₂ (9).

In different experimental models, an overexpression of either SM22 (11), apolipoprotein J (12), osteonectin (13), or fibronectin (14) is induced by extracellular addition of transforming growth factor- β 1 (TGF- β 1). Moreover, incubation of HDFs with TGF- β 1 triggers the appearance of a senescent-like morphology (15, 16) and growth arrest (17).

Two main arguments favor the hypothesis that oxidative stress-induced premature senescence could be triggered by a pRb-mediated TGF- β 1 overexpression. First, it has been shown that ATF-2 transcription factor-hypophosphorylated Rb complexes transactivate the TGF- β promoter (18). Second Rb remains hypophosphorylated at least for several weeks after H₂O₂-induced SIPS (10).

Studies were performed with stable IMR-90 HDF transfectants expressing the E6 or E7 protein of the human papilloma virus 16, which namely, respectively, bind and facilitate the degradation of p53 and Rb. IMR-90 HDFs, whether wild types or expressing the E6 gene, developed a senescent morphology after subcytotoxic treatment under H₂O₂, while the cells expressing E7 gene did not. Furthermore, deletion of amino acids 21–24 as well as mutations at positions 24–26, which eliminated the binding of E7 to Rb (19), brought back the senescent morphology after H₂O₂ stress (10). Taken together, these first experiments suggested that Rb is necessary in the induction of SIPS.

These arguments led to the hypothesis that the E7-mediated disappearance of Rb makes impossible the formation of the ATF-2-Rb complexes, which in turn does not allow the stress-induced transcription of TGF- β 1 and the subsequent overexpression of apolipoprotein J, osteonectin, fibronectin, and SM22 mRNA.

The aim of this work was first to test the involvement of TGF- β 1 in the appearance of SIPS as far as changes in mor-

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¹ The abbreviations used are: HDF, human diploid fibroblast; RT-PCR, reverse transcription polymerase chain reaction; SIPS, stress-induced premature senescence; *t*-BHP, *tert*-butylhydroperoxide; SA, senescent-associated; β -gal, β -galactosidase; Rb, retinoblastoma protein; TGF- β 1, transforming growth factor- β 1; MEM, minimal essential medium; FBS, fetal bovine serum; FS, fibroblast skin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TABLE I
Primers used for RT-PCR

Genes	Positions	Sequences
	<i>bp</i>	
Osteonectin	181–199	5'-ctg tgg gag cta atc ctg-3'
	765–783	5'-ggg tgc tgg tcc agc tgg-3'
Fibronectin	360–378	5'-gat tgc ctg ttc tgc ttc-3'
	502–520	5'-ttg ggt gac ttt cct act-3'
Apolipoprotein J	69–87	5'-cgg ggt gaa aca gat aaa g-3'
	373–391	5'-tgc ggt cac cat tca tcc a-3'
SM22	249–267	5'-tgg cgt gat tct gag caa-3'
	470–488	5'-ctg cca agc tgc cca agg-3'
GAPDH	333–353	5'-cgt ctt cac cat gga ga-3'
	612–622	5'-cgg cca tca cgc cca cag ttt-3'
TGF- β 1	Catalog number 01923–42 (Biagnostik, Göttingen, Germany)	

phology, SA β -gal, and mRNA level of these four genes are concerned. We tested whether these biomarkers appear after incubations of IMR-90 HDFs with TGF- β 1. Then we tested whether TGF- β 1 mediates the appearance of these biomarkers in SIPS.

Furthermore, we tested whether stable IMR-90 HDF transfectants expressing the E7 or E6 viral protein would overexpress TGF- β 1 after stress. Last, if the E7- or E6-mediated inhibition of the stress-induced overexpression of TGF- β 1 took place, we wished to test whether E7 or E6 would inhibit the stress-induced overexpression of fibronectin, osteonectin, apolipoprotein J, and SM22.

MATERIALS AND METHODS

Cell Culture—Fetal lung IMR-90 HDFs (European Cell Culture Collection, Strasbourg, France) were grown in MEM + 10% (v/v) of fetal bovine serum (FBS). Human skin FS AG04437B HDFs (ATCC, Manassas, VA) were grown in Basal Medium of Eagle + 10% (v/v) of fetal bovine serum. Confluent IMR-90 HDFs under 45–50% of *in vitro* proliferative lifespan were exposed for 2 h to 150 μ M H₂O₂ diluted in MEM + 10% FBS. FS AG04437B HDFs were exposed to several concentrations of H₂O₂ diluted in Basal Medium of Eagle + 10% FBS. The cells were, respectively, rinsed with MEM or Basal Medium of Eagle and given respective fresh medium + 10% FBS. TGF- β 1 (R & D Systems) was diluted in culture medium plus serum to stimulate IMR-90 HDFs for 72 h.

Neutralization of the TGF- β 1 Receptor II and the TGF- β 1 Itself—TGF- β RII receptor of IMR-90 HDFs was neutralized using a specific antibody from R & D Systems (AF-241NA) diluted at 10 μ g/ml of MEM + 10% FBS. The antibody against TGF- β 1 (R & D Systems, MAB-240) was diluted at 3 μ g/ml of MEM + 10% FBS. The medium containing the neutralization antibody was replaced every day for 3 days.

Infection with Recombinant Human Papillomavirus E6 or E7 Retroviral Constructs—The human papillomavirus type 16 E6 or E7 retrovirus producing cells were obtained from the ATCC (Manassas, VA). Exponentially growing IMR-90 HDFs (CPD 16.8) were infected for 4 h with retroviruses carrying PLXSN vector without insert (termed control PLXSN vector) or carrying human papillomavirus E6 or E7 in 6 ml of MEM containing 10% FBS and 4 μ g/ml polybrene. The volume of medium was doubled for overnight incubation followed by medium change. The HDFs expressing the E6 or E7 gene were selected by exposure to 500 μ g/ml of G418 (Life Technologies, Inc.).

RNA Isolation and Semiquantitative RT-PCR—Cells were harvested and pelleted. Total RNA was extracted using a total RNA isolation kit (PharMingen). Semiquantitative RT-PCRs were achieved in the exponential linear zone of amplification for each gene studied in one step with the Access RT-PCR kit (Promega) in presence of [α -³²P]dideoxycytosine (0.025 μ CI per reaction). RT-PCRs were performed in the common exponential range of the PCR amplification using 50 and 100 ng of total RNA in 50 μ l containing 5 units of avian myeloblastosis virus reverse transcripts and 5 units of *Pfu* polymerase.

We used GAPDH mRNA level as reference. GAPDH mRNA level was checked to be stable in all experimental conditions tested (specific primers for GAPDH and each gene tested in Table I). RT-PCR products were electrophoresed on a 5% (w/v) polyacrylamide gel and analyzed (Instant Imager, Packard Instrument Co.). The dried gels were exposed to β -max films (Amersham Pharmacia Biotech, Anvers, Belgium). Neg-

ative controls were performed without RNA. A PCR without reverse transcription was always performed on RNA, prior to reverse transcription, to check for DNA contamination. The sequences of the RT-PCR products were checked for amplification specificity. The results were expressed as mean value \pm S.D. on three independent experiments.

RNAse Protection Assay—RNAse protection assays were performed following the instruction of the multi-probe hCK-3 (catalog number 45033P, PharMingen). The antisense probes were synthesized with bacteriophages T7 RNA polymerase in the presence of [α -³²P]UTP and purified by phenol-chloroform extraction. The probe set was hybridized in excess to specific target mRNAs in solution. Free probes and single-stranded RNA were digested by RNases. The remaining protected RNA/probes were resolved in denaturing polyacrylamide gels and quantified by Instant Imager (Packard Instrument Co.). The results were expressed as percentages of the corresponding values found in the non-stressed IMR-90 HDFs transfected with the control PLXSN without insert. The results of RNAse protection assays are given as mean value \pm S.D. on three independent experiments.

Senescent-associated β -Galactosidase Activity—The proportion of HDFs positive for the SA β -gal activity was determined as described by Dimri *et al.* (20). The cells were plated at a low density and fixed with 2% formaldehyde and 0.2% glutaraldehyde. The presence of the SA β -gal activity was determined by incubation with 1 mg/ml of solution of 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside in 0.04 M citric acid/sodium, 0.005 M K₃FeCN₆, 0.005 M K₄FeCN₆, 0.15 M NaCl, 0.002 M MgCl₂ diluted in phosphate-buffered saline (pH 6). The cells were rinsed twice with phosphate-buffered saline and washed with methanol. The results were expressed as mean value \pm S.D. on three independent experiments.

Enzyme-linked Immunosorbent Assay—The active TGF- β 1 present in the culture medium was quantified using the TGF- β 1 Emax ImmunoAssay System of Promega (Madison, WI). For each sample the quantification was made twice. The results were expressed as mean value \pm S.D. on three independent experiments.

Analysis of the Rb Phosphorylation Status by Western Blot—The cells were washed twice with ice-cold phosphate-buffered saline and lysed on ice with 700 μ l of lysis buffer (25 mM HEPES, 0.3 M NaCl, 1.5 mM MgCl₂, 20 mM β -glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1% Triton X-100, 10% glycerol, 10 μ g/ml each aprotinin and leupeptin, 100 mM NaF, 1 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride). After lysis, the material was homogenized several times through a 21-gauge needle and centrifuged at 13,000 \times g for 20 min at 4 $^{\circ}$ C. Samples of 20 μ g of protein were electrophoresed on 7.5% SDS-polyacrylamide gels. The proteins were transferred overnight on Immobilon-P membrane (Millipore). Rb was detected with anti-Rb (c-15, Santa Cruz Biotechnology) antibody and thereafter with horseradish peroxidase-linked secondary antibody. The bands were visualized after incubation with chemoluminescent substrates using ECL detection kit (RPN 2106, Amersham Pharmacia Biotech, Anvers, Belgium).

RESULTS

SA β -Galactosidase Activity and mRNA Level of Four Senescence-associated Genes after Stimulation with TGF- β 1—IMR-90 HDFs were stimulated for 3 days with 1–10 ng/ml of human TGF- β 1 diluted in culture medium + 10% FBS. The proportion of HDFs positive for the SA β -gal activity was determined in three independent experiments. A significant dose-dependent increase in the proportion of SA β -gal-positive HDFs was obtained, ranging from a 103 to a 160% increase, when the absolute value of 15% of SA β -gal-positive cells in controls cells was considered as 100%. In comparison, the proportion of SA β -gal-positive cells at 72 h after treatment with 150 μ M H₂O₂ for 2 h reached an absolute value of 55% (Fig. 1).

Semiquantitative RT-PCR was used to determine the mRNA level of four genes that undergo a senescence-related overexpression: fibronectin, apolipoprotein J, SM22, and osteonectin. After 72 h of stimulation with 1–10 ng/ml TGF- β 1, the steady-state level of mRNA of the four genes was found to be increased when compared with nontreated cells. A dose-dependent overexpression was obtained for fibronectin, osteonectin, and SM22. Two transcripts of fibronectin were detected and displayed a dose-dependent overexpression, reaching a 2.15-fold increase at 10 ng/ml TGF- β 1. SM22 overexpression was maximum at 10 ng/ml TGF- β 1, with a 2.30-fold increase in mRNA

level. Osteonectin was similarly overexpressed at the three TGF- β 1 concentrations with an increase ranging from 1.48- to 1.70-fold. Apolipoprotein J was maximally overexpressed at 1 ng/ml TGF- β 1, with a 1.90-fold increase and minimally overexpressed at 10 ng/ml TGF- β 1 (Fig. 2).

These data suggest that TGF- β 1 triggers the appearance of SA β -gal in IMR-90 HDFs as well as the overexpression of at least four genes that show a senescence-related overexpression. The next step was to know whether TGF- β 1 is overexpressed during the establishment of H₂O₂-induced premature senescence.

Expression of TGF- β 1 in H₂O₂-induced Premature Senescence—At first, semiquantitative RT-PCR was performed on mRNA extracted at 72 h after 2-h exposures of IMR-90 HDFs to 150 μ M H₂O₂. When compared with nontreated cells, a 1.8-fold increase in the mRNA level of TGF- β 1 was obtained (Fig. 3). RNase protection assay showed that TGF- β 2 was not overexpressed at 72 h after stress. TGF- β 3 mRNA was not detected by RNase protection assay in any condition. RNase protection assay also confirmed TGF- β 1 overexpression (not shown). Similar experiments were performed on FS AG04437B HDFs exposed to four H₂O₂ concentrations ranging from 150 to 300 μ M. Higher H₂O₂ concentrations were used, since cytotoxicity curves showed that FS AG04437B HDFs are much more resistant to H₂O₂ (not shown). The amount of TGF- β 1 mRNA at 72 h after stress raised from 130% at 150 μ M H₂O₂ to 190% at 300 μ M in H₂O₂ when compared with the 100% control values (not shown). A second set of semiquantitative RT-PCR was performed on IMR-90 HDFs mRNA extracted from independent

cultures at various times after exposures to H₂O₂. The mRNA steady-state level of TGF- β 1 decreased during the first 4 h after stress and increased up to 140% at 24 h. The level of mRNA returned to the basal level at 36 h after stress and increased again to 150% at 48 h.

We used enzyme-linked immunosorbent assay to detect active TGF- β 1 protein released in the culture medium during the 48 h following the stress. The level of TGF- β 1 protein was also measured in non-H₂O₂-exposed control cells. At each time after the stress, the amount of active TGF- β 1 released by the

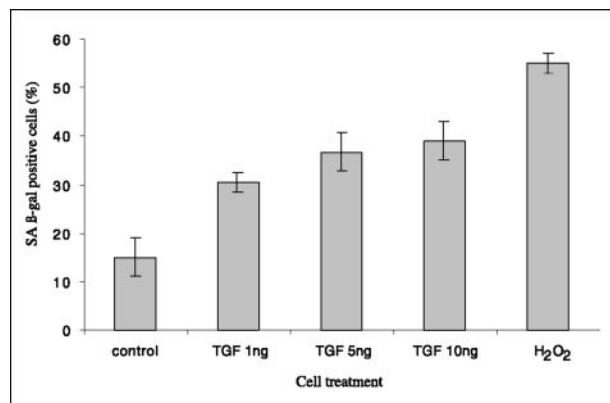


FIG. 1. Percentage of IMR-90 HDFs positive for SA β -galactosidase activity after 3 days of stimulation with TGF- β 1 at 1, 5, and 10 ng/ml.

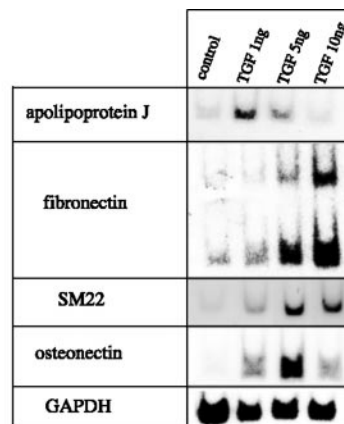


FIG. 2. Steady-state level of fibronectin, apolipoprotein J, osteonectin, and SM22 mRNA after 3 days of stimulation of IMR-90 HDFs with TGF- β 1 at 1, 5, and 10 ng/ml. *Top panel*, autoradiography after migration of the products obtained by semiquantitative RT-PCR performed on fibronectin, osteonectin, apolipoprotein J, and SM22 mRNA with GAPDH mRNA as reference. *Bottom panel*, quantification performed at 72 h after the stress.

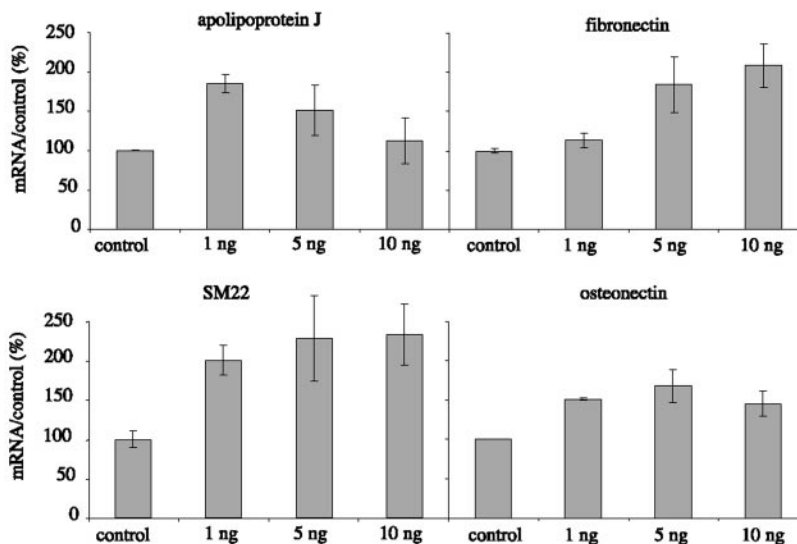


FIG. 3. Steady-state level of the TGF- β 1 mRNA at various times after exposure to 150 μ M H₂O₂. Top panel, autoradiography after migration of the products obtained by semiquantitative RT-PCR performed on TGF- β 1 mRNA with GAPDH mRNA as reference. -, RT-PCR without RNA. Bottom panel, quantification performed at 72 h after the stress.

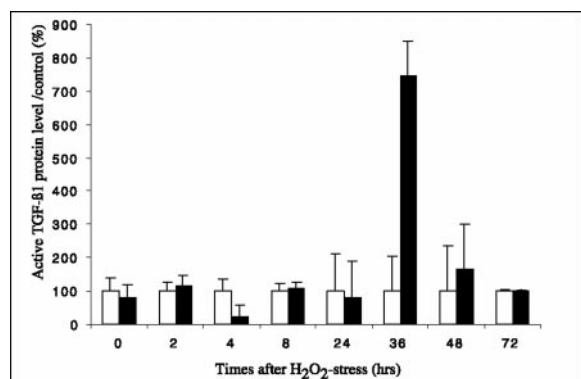
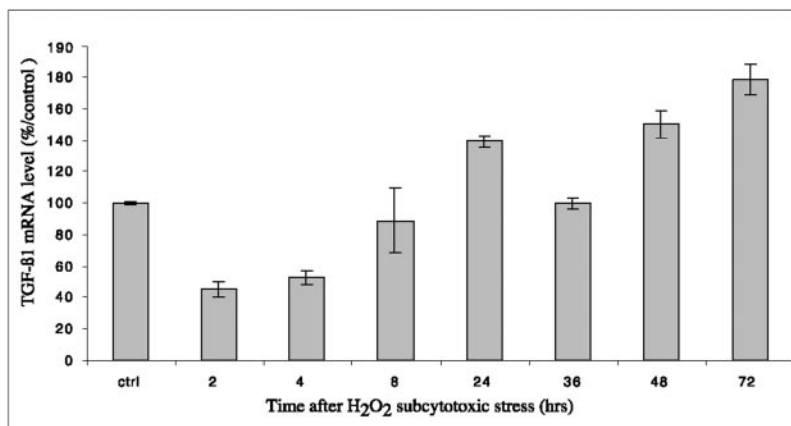
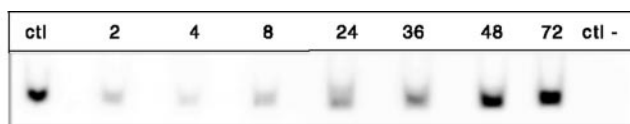


FIG. 4. Quantification of the active TGF- β 1 released in the culture medium after the H₂O₂ stress and in control cells. The results are expressed as percentage of the amount of active TGF- β 1 present in the control at each time after the stress.

stressed cells was expressed as percentage of the amount released by the control cells. Surprisingly, the level of released TGF- β 1 underwent a 7.5-fold increase from 24 to 36 h after the stress (Fig. 4). This increase disappeared from 36 to 48 h after the stress. Given these spectacular results, we wished to neutralize the TGF- β receptor II (TGF- β RII) and TGF- β 1 to know whether this would influence the appearance of the biomarkers of stress-induced premature senescence.

Neutralization of TGF- β RII and TGF- β 1—IMR-90 HDFs were stressed with 150 μ M H₂O₂ for 2 h. They were incubated with culture medium + 10% FBS + antibodies against either TGF- β 1 (TGF- β 1-Ab) or TGF- β R II (TGF- β RII-Ab). New culture medium + 10% FBS + 10 μ g/ml TGF- β RII-Ab or 3 μ g/ml TGF- β 1 Ab was provided to the cells for three successive days after the stress.

The non-H₂O₂-exposed HDFs incubated with TGF- β RII-Ab or TGF- β 1-Ab had a normal morphology. Senescent-like morphology was obtained at 72 h after H₂O₂ treatment as shown previously (4). No senescent-like morphology was obtained in the stressed HDFs incubated with anti-TGF- β II or TGF- β 1-Ab for 3 days after the stress (Fig. 5).

We determined the percentage of HDFs positive for the SA β -gal activity in these different conditions. After incubation with TGF- β 1- or TGF- β RII-Abs for 72 h, no significant modification of the percentage of SA β -gal-positive cells was observed in normal conditions when compared with control cells (no stress, no antibody).

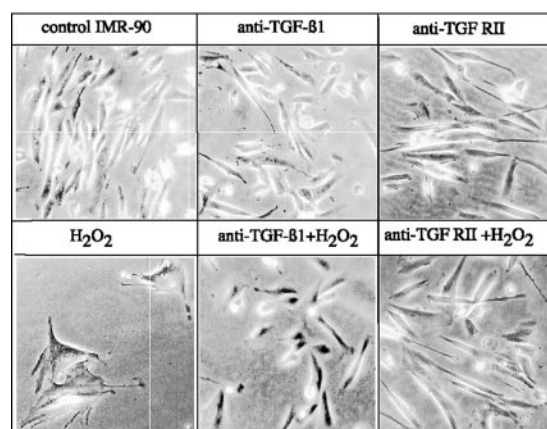


FIG. 5. Effect of anti-TGF- β 1 or anti-TGF- β receptor II antibody on the morphology of IMR-90 HDFs at 72 h after exposure to 150 μ M H₂O₂ stress or not.

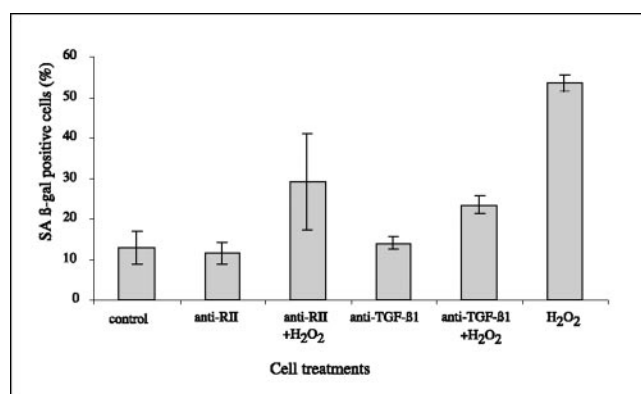


FIG. 6. Effect of anti-TGF- β 1 or anti-TGF- β receptor II antibody on the percentage of IMR-90 HDFs positive for the SA β -galactosidase activity at 72 h after 150 μ M H₂O₂ stress.

The cells incubated with anti-TGF- β 1- or -TGF- β RII-Abs for 3 days after stress displayed a significantly decreased proportion of SA β -gal-positive cells in comparison with the H₂O₂-treated cells. Indeed, only a 2.40-fold and a 1.70-fold increase in the percentage of SA β -gal-positive cells was observed, respectively, in the HDFs incubated with anti-TGF- β RII- or anti-TGF- β 1-Abs after stress, while a 4.40-fold increase was observed in the H₂O₂-treated HDFs, which were not incubated

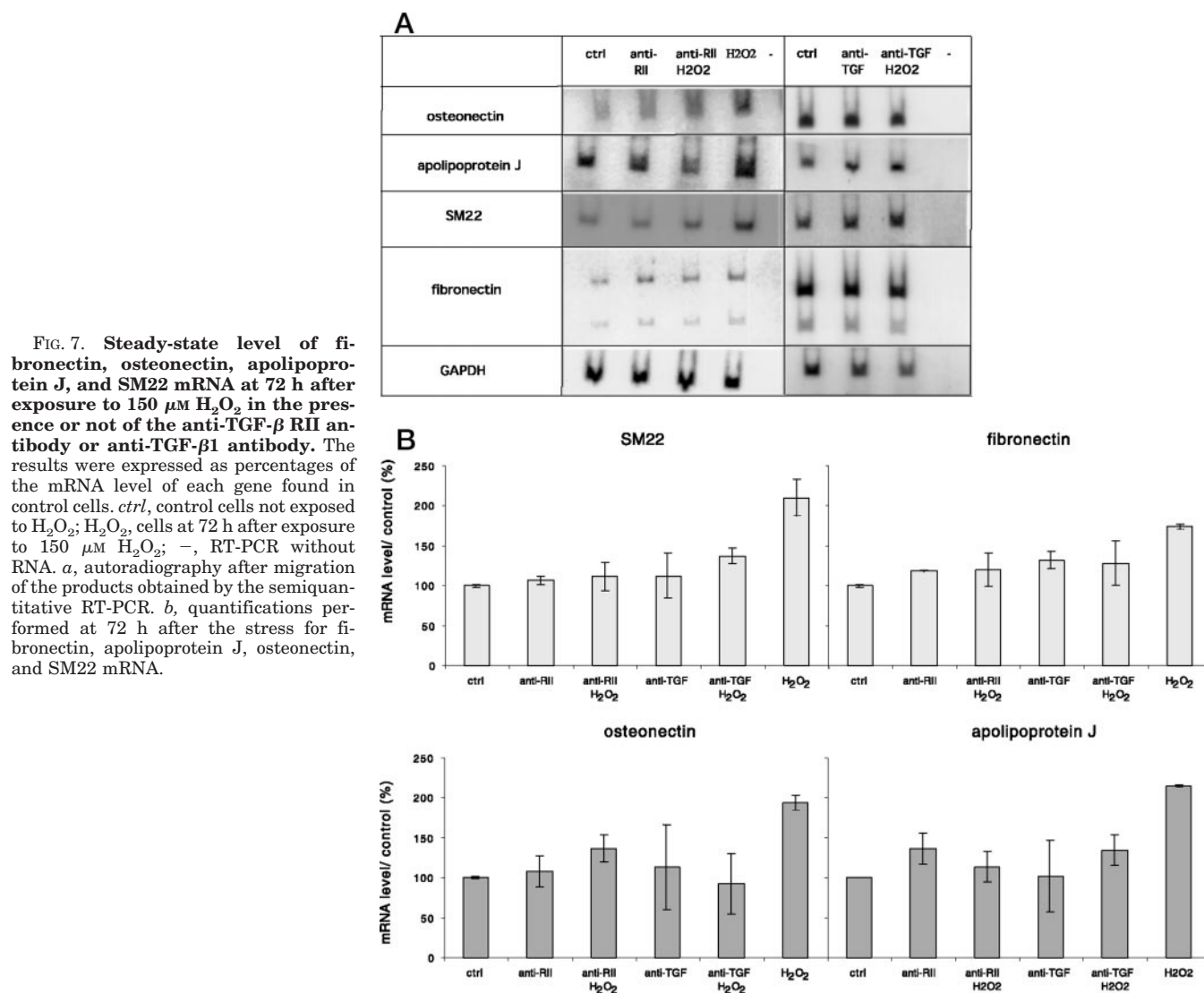


FIG. 7. Steady-state level of fibronectin, osteonectin, apolipoprotein J, and SM22 mRNA at 72 h after exposure to 150 μ M H₂O₂ in the presence or not of the anti-TGF- β RII antibody or anti-TGF- β 1 antibody. The results were expressed as percentages of the mRNA level of each gene found in control cells. *ctrl*, control cells not exposed to H₂O₂; H₂O₂, cells at 72 h after exposure to 150 μ M H₂O₂; -, RT-PCR without RNA. *a*, autoradiography after migration of the products obtained by the semiquantitative RT-PCR. *b*, quantifications performed at 72 h after the stress for fibronectin, apolipoprotein J, osteonectin, and SM22 mRNA.

with one of these Abs (Fig. 6).

Last, we compared the steady-state levels of mRNA of fibronectin, osteonectin, SM22, and apolipoprotein J. First, we confirmed that these steady-state levels were increased in replicative senescence and H₂O₂-induced premature senescence (not shown) as shown by Dumont *et al.* (9). For all the genes tested, a much lower overexpression was observed when the H₂O₂-treated cells were incubated with TGF- β RII- or TGF- β 1-Abs (Fig. 7, *a* and *b*).

Since TGF- β 1 mediates the appearance of several biomarkers of stress-induced premature senescence, we wished to have clues on the regulation of the stress-induced TGF- β 1 overexpression.

Effects of the E6 and E7 Proteins on the Stress-induced Increase of TGF- β 1 mRNA—We exposed IMR-90 HDFs transfected with a retroviral PLXSN control vector, PLXSN + E6 cDNA or PLXSN + E7 cDNA, to 150 μ M H₂O₂ for 2 h in three independent experiments. Total RNA was extracted at 72 h after the stress. At first, we have shown by Western blots (10) that IMR-90 HDFs transfected with E6 or E7 cDNA have a very low respective level in p53 and Rb proteins. Semiquantitative RT-PCR was performed using GAPDH as reference gene, whose transcription level was checked to be stable in IMR-90 HDFs transfected by E6, E7, exposed to H₂O₂ or not (not shown). A 1.55-fold increase in TGF- β 1 mRNA level was shown at 72 h after the stress in HDFs transfected with a retroviral

PLXSN control vector, as shown herein in nontransfected HDFs exposed to H₂O₂.

In normal conditions (no H₂O₂ treatment), the level of TGF- β 1 mRNA raised by about 50% in IMR-90 HDFs transfected with E6 or E7. More interestingly, the TGF- β 1 mRNA level of IMR-90 HDFs + E6 was much higher in H₂O₂-stressed cells when compared with nonstressed cells, while this level was not further increased in H₂O₂-stressed IMR-90 + E7 HDFs when compared with nonstressed IMR-90 + E7 HDFs (Fig. 8). Using enzyme-linked immunosorbent assay, we measured the release of active TGF- β 1 at increasing times after treatment of IMR-90 + E7 HDFs with H₂O₂. No increased release of TGF- β 1 level was observed in IMR-90 + E7 HDFs and in IMR-90 + E6 HDFs treated with H₂O₂ (data not shown). These results suggest that p53 and Rb are necessary for the stress-induced increase in the release of TGF- β 1.

Moreover, Western blotting was carried out to analyze the phosphorylation status of Rb at 72 h after stress. Rb were found to be hypophosphorylated (Fig. 9), confirming the results obtained by Chen *et al.* (10) after H₂O₂ and by Dumont *et al.* (2000) after *tert*-butylhydroperoxide stress (9). Rb must be hypophosphorylated to interact with ATF-2 and transactivate the TGF- β promoter. Therefore our results suggest that hypophosphorylated Rb protein mediates the stress-induced overexpression of TGF- β 1. Further experiments are being performed to confirm the role of Rb in this overexpression.

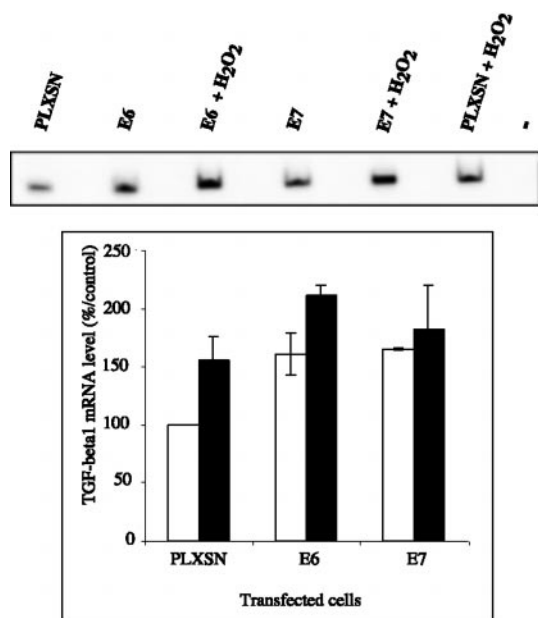


FIG. 8. Steady-state level of the TGF- β 1 mRNA in IMR-90 + E6 or IMR-90 + E7 HDFs. Semiquantitative RT-PCR was performed on total RNA extracted from IMR-90 + control PLXSN vector, IMR-90 + E6 HDFs, and IMR-90 + E7 HDFs at 72 h after exposure to 150 μ M H_2O_2 or not. White columns, cells not exposed to H_2O_2 ; black columns, cells exposed to 150 μ M H_2O_2 . -, RT-PCR without RNA.

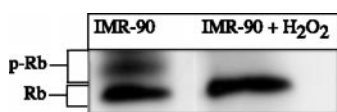


FIG. 9. Phosphorylation status of the Rb protein at 72 h after exposure of IMR-90 HDFs to 150 μ M H_2O_2 . p-Rb, phosphorylated Rb; Rb, hypophosphorylated Rb.

Regulation of the Expression of Senescence-related Genes in H_2O_2 -treated Cells—According to the hypothesis mentioned in the introduction, an E7-mediated inhibition of the stress-induced overexpression of TGF- β 1 should lead to an inhibition of the stress-induced overexpression of fibronectin, osteonectin, apolipoprotein J, and SM22. We performed semiquantitative RT-PCR to evaluate the mRNA level of these four genes. We exposed normal IMR-90 HDFs, IMR-90 + control PLXSN vector, IMR-90 + E6 HDFs, and IMR-90 + E7 HDFs to 150 μ M H_2O_2 for 2 h and extracted total RNA at 72 h after the stress. The results of the quantifications were expressed as percentages of the value recorded for the nonstressed IMR-90 HDFs + control PLXSN vector. Similar results were obtained for normal IMR-90 HDFs transfected or not with control PLXSN vector.

We confirmed that all four genes were overexpressed at 72 h after H_2O_2 stress in IMR-90 cells as shown previously (9). The stress-induced overexpression of osteonectin and apolipoprotein J observed in the normal HDFs disappeared in IMR-90 + E7 HDFs. The stress-induced overexpression of SM22 was highly decreased, by more than 60%, in H_2O_2 -treated IMR-90 + E7 HDFs when compared with the H_2O_2 -treated IMR-90 HDFs transfected or not with control PLXSN vector. In IMR-90 + E7 HDFs, a 2-fold reduction in fibronectin mRNA level was found in the nontreated cells. No stress-induced overexpression of fibronectin was found in these cells (Fig. 10 a and b).

When IMR-90 + E6 cells were stressed with H_2O_2 , the overexpression of osteonectin disappeared. E6 caused a marked down-regulation of the mRNA of fibronectin in nonstressful conditions.

These data show that E7 regulates the stress-induced ex-

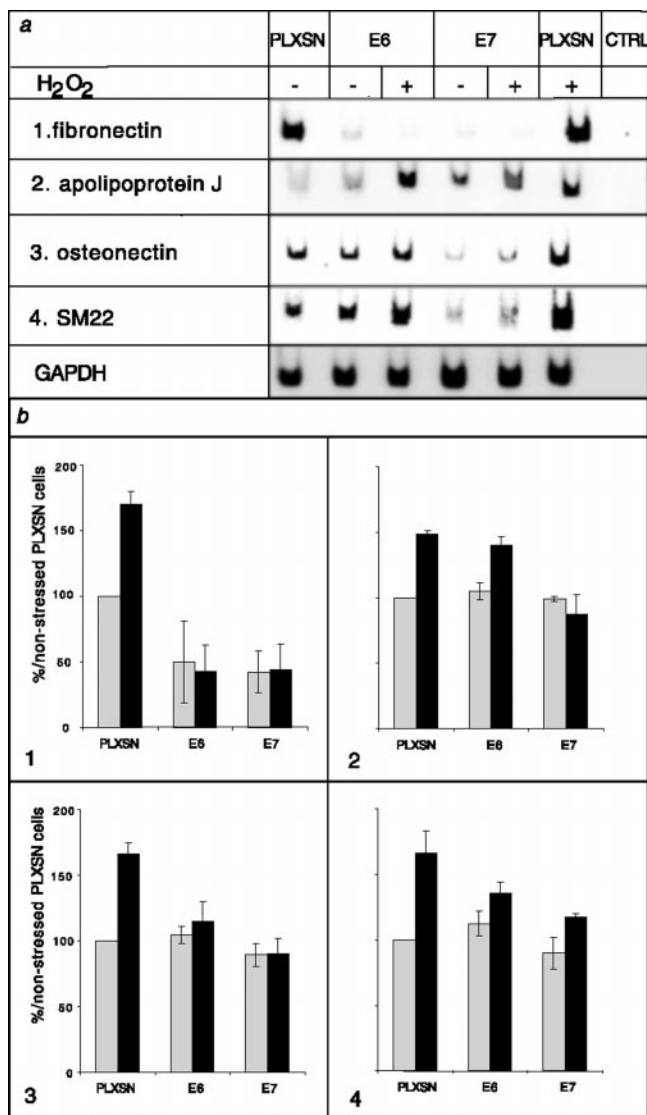


FIG. 10. Steady-state level of fibronectin, apolipoprotein J, osteonectin, and SM22 mRNA in IMR-90 + E7 and IMR-90 + E6 HDFs at 72 h after exposure to 150 μ M H_2O_2 . a, autoradiography after migration of the products obtained after semiquantitative RT-PCR performed on fibronectin, apolipoprotein J, osteonectin, and SM22 mRNA with GAPDH mRNA as reference. IMR-90 + control PLXSN vector, IMR-90 + E6 HDFs, and IMR-90 + E7 HDFs were exposed or not to 150 μ M H_2O_2 for 2 h. -, RT-PCR without RNA. b, quantifications performed at 72 h after the stress for fibronectin (panel 1), apolipoprotein J (panel 2), osteonectin (panel 3), and SM22 (panel 4) mRNA of cells exposed (black columns) or not (gray columns) to H_2O_2 .

pression of all four genes studied. These results suggest that Rb is involved in the regulation of the stress-induced and TGF- β 1-mediated overexpression of these genes. As explained in the introduction, Rb is also necessary for the morphological changes observed in H_2O_2 -induced SIPS (10). Work in progress is aimed at confirming this involvement of Rb.

DISCUSSION

The concept of SIPS is widely accepted (1). However, the mechanisms responsible for its occurrence remain poorly understood.

Many biomarkers of replicative senescence have been shown to be induced in SIPS (9). A typical morphology, senescence-associated β -galactosidase activity and overexpression of fibronectin, osteonectin, SM22, and apolipoprotein J are among these biomarkers.

Several observations were in favor of the involvement of

TGF- β 1 in SIPS. First, Rb becomes hypophosphorylated in normal senescence, in SIPS, and also after stimulation of human monocytic JOSK-1 leukemia cells line (21) or mesengial cells (22) with TGF- β 1. Second, interactions between ATF-2 and hypophosphorylated Rb have been shown to trigger the transactivation of the promoter of TGF- β (18). Third, the overexpression of each of the four senescence-associated genes studied herein has been shown to be controlled by TGF- β , in four respective models (11–14, 23).

In this work, we show that TGF- β 1 induces a senescence-like morphology, senescence-associated β -galactosidase activity, and overexpression of these four senescence-associated genes. Then we have shown that TGF- β 1 mRNA is increased at 72 h after subcytotoxic H₂O₂ stress in conditions where SIPS is triggered. Further experiments have shown that this increase in mRNA level starts at 24 h after the stress. In this study, a peak of release of active TGF- β 1 protein in the culture medium was observed at 36 h after stress, suggesting a complex regulation taking place either at the level of RNA stability or post-transcriptionally. Since other peaks of TGF- β 1 protein release might exist during the 72 h necessary for SIPS to take place, we incubated the cells with TGF- β RII- or TGF- β 1-Abs for 72 h after the stress. Our results strongly suggest that TGF- β 1 mediates the induction of the senescence-like morphology, senescence-associated β -galactosidase activity, and the overexpression of the four senescence-associated genes (fibronectin, osteonectin, SM22, and apolipoprotein J) studied.

Previous work (24) suggested a possible involvement of TGF- β 1 in the morphological changes taking place in the γ -radiation-induced growth arrest. Here, we give evidence that TGF- β 1 controls the appearance of several biomarkers of cellular senescence in conditions leading to SIPS. We also provide clues for the mechanism of stress-induced overexpression of the TGF- β 1 gene. We show that the stress-induced protein release of TGF- β 1 disappeared in IMR-90 + E6 HDFs and IMR-90 + E7 HDFs, suggesting that p53, Rb, or other partners of Rb signaling pathway regulate TGF- β 1 expression in stressful conditions. Moreover, the results show also that E7 inhibits the stress-induced overexpression of fibronectin, osteonectin, SM22, and apolipoprotein J and therefore reinforce the notion that Rb might control the overexpression of these senescence-associated genes through TGF- β 1 overexpression. Further experiments are necessary to confirm this likely role of Rb in the overexpression of TGF- β 1 in conditions leading to SIPS.

Nevertheless, it has been shown, in the same experimental system, that deletion of amino acids 21–24 of E7 cDNA, as well as mutations at positions 24–26, which eliminate the binding of E7 to Rb, allow the appearance of SIPS, while wild type E7

does not (10). This result reinforces the potential role of Rb in the appearance of various biomarkers of SIPS. Further experiments are needed to know whether TGF- β 1 controls the appearance of other biomarkers of SIPS. It would also be interesting to know whether HDFs, which are unable to express TGF- β 1, also undergo SIPS after subcytotoxic stresses and/or postpone their replicative senescence in normal conditions. Last, other studies could be aimed at finding out whether the stress-induced overexpression of TGF- β 1 found in SIPS is triggered by DNA damage generated by H₂O₂, which is likely to be the case.

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