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Effect of Radiation and Repeated Sub-culturing on the Transforming Growth Factor-β1 Signaling Pathway in FRTL-5 Cells

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Abstract. *Background/Aim: Fisher rat thyroid cells (FRTL-5) display increased proliferation, reduced follicularization and decreased thyroxin release with repeated sub-culturing. These changes occur earlier and more rapidly following exposure to ionizing radiation. We hypothesized that altered transforming growth factor-β1 (TGF-β1) signaling contributes to these differences. Materials and Methods: Assessments included FRTL-5 cell growth rate and quantification of TGF-β1 ligand and receptors. The levels and activity of Smads2, 3 and 4 were measured by western blotting and the ability of TGF-β1 to regulate cyclin A and plasminogen activator inhibitor type 1 (PAI-1) activity was assessed using transfection assays. Results: TGF-β1 production increased after radiation but returned to control levels after repeated sub-culturing. There was no difference in TGF-β1 levels between un-irradiated cells at low versus high-passage number. TGF-β1 receptors and basal levels of Smads2, 3 and 4 remained unchanged. However, there were significant changes in cell proliferation, TGF-β1-mediated Smads2 and 3 activation and in TGF-β1's ability to regulate cyclin A and PAI-1 transcription in irradiated and repeatedly sub-cultured cells ($p<0.05$). Conclusion: Collectively, these results support the conclusion that alterations in the TGF-β1 pathway contribute to phenotypic changes in FRTL-5 cells as a function of passage number and radiation.*

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The transforming growth factor-β1 (TGF-β1) signaling pathway is important not only in normal cell growth inhibition but also in progression of cells to a malignant phenotype, cancer metastasis and fibrosis associated with radiotherapy. When normal cells/tissues are exposed to ionizing radiation, an early measurable response is to activate repair pathways so as to regain homeostasis (1). A key aspect of repair is to arrest the cell cycle, thus inhibiting cell division, so that there is time to repair the damage caused by radiation. One of the major molecules involved in control over cell-cycle progression is TGF-β1 (1-3). If the cells lose their normal response to cell-cycle arrest under TGF-β1 control, damage caused by radiation may go un-repaired. This could prompt the development of genomic instability in these cells (4). Radiation may disrupt normal protective machinery that leads to uncontrolled cell proliferation and display of abnormal properties (4). One of the reasons resistance to TGF-β1 can develop is because of a defect in the TGF-β1 signaling pathway itself (2, 3). For TGF-β1 signaling to occur normally, the ligand first binds to transforming growth factor-beta receptor type II (TGF-βRII) and subsequently to transforming growth factor-beta receptor type I (TGF-βRI) (serine/threonine kinase receptors for TGF-β1) (5-8). Both receptors need to be present in equimolar amounts for normal signaling to occur (2, 9). The signaling continues from the receptor to the nucleus when activated TGF-βRI phosphorylates Smad2 and 3, triggering their activation and translocation (5, 6). The interaction of Smad2/3 with Smad4 forms a complex that, along with other proteins, migrates to the nucleus and affects transcriptional regulation (5, 6, 8, 10). In most epithelial cells, cell-cycle arrest caused by TGF-β1 occurs in the G_1 phase, thus preventing the G_1 to S phase transition (2, 10). It does so *via*

Smad-mediated transcriptional activation of the cyclin-dependent kinase inhibitors (CKIs), which prevent the synthesis and activation of cyclins (*e.g.* cyclin A) and cyclin-dependent kinases (CDKs) (10-13). However, a defect in any of the components of the signaling pathway can result in cells that are no longer responsive to growth inhibition by TGF-β1 (2-4).

Based on the findings above and on our previous observations with the FRTL-5 cells, it seems likely that a similar phenomenon occurs in these cells. Normally, early passage FRTL-5 cells spontaneously develop thyroid follicles, release thyroxin, express thyroid stimulating hormone (TSH) receptors and require TSH for growth in tissue culture (14-16). However, when the cells were sub-cultured more than 15 times, their morphology changed and their growth rate increased relative to earlier passages. The late-passage cultures had an accelerated doubling-time such that by passage 25 (P25) it was only half that of lower passage cultures (14). There was also a reduction (~20%) in the fraction of cells organized into follicles and thyroxin release as the cultures were growth-expanded more than 15 times (14). An in-depth study conducted in our laboratory revealed that a spontaneous mutation in connexin 32 (Cx32), the only gap junction protein produced in FRTL-5 cells, contributed to the differences observed in the cellular properties after repeated sub-culturing. This mutation resulted in the formation of a non-functional truncated form of Cx32, which caused a reduction in gap-junction communication among the cells/follicles (14). These Cx32-defective late-passage cells were more sensitive to gamma radiation (^{60}Co) than earlier passages. This was attributed to their diminished functional (follicular) organization that increased with repeated sub-culturing. The dysfunctional organization prevented the cells/cultures from communicating with each other and, thus, maintaining homeostasis after the radiation insult (16). Based on these findings, we postulated that radiation exposure could accelerate the onset of morphological changes that we detected in FRTL-5 cells as they were repeatedly growth-expanded.

In addition, our ongoing studies with FRTL-5 cells led us to question whether the altered properties exhibited by these cells with repeated sub-culturing were due to more than the mutation in Cx32. Our results indicated that TGF-β1 signaling may be altered. Thus, the current goal was to examine the TGF-β1 signaling pathway to determine whether defects were contributing to the changes recorded in this cell line as a function of repeated sub-culturing and whether these changes were accelerated by exposure to radiation.

In the present study, changes in cell growth rate and levels of TGF-β1 and its receptors were quantified in early (sub-cultured 7-8 times) and late (sub-cultured 18 times) passage FRTL-5 cultures following exposure to radiation. We examined whether repeated sub-culturing and/or radiation exposure induced changes in the basal and phosphorylation levels of Smad proteins, which are important in TGF-β1 signaling. Also, we tested whether the role of TGF-β1 transcriptional activation of cyclin A and plasminogen activator inhibitor-1 (PAI-1) were altered as a result of the age of the cultures and/or exposure to radiation.

Materials and Methods

Cell culture. FRTL-5 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were at an unknown passage number when received. Passage numbers were, therefore, recorded from the time of receipt, as the letter P followed by the appropriate number (*e.g.* P1) and will be referred to as such hereafter. The cells were growth-expanded and at various passages stored frozen in liquid nitrogen. Cells used in these experiments were mainly derived from our P7 FRTL-5 stocks (unless otherwise noted), brought up in DMEM/F12 medium (50:50 v/v, Life Technologies, Gaithersburg, MD, USA), supplemented with 5% calf serum (Summit Biotechnologies, Fort Collins, CO, USA), 2mM glutamine and a six-hormone mix (10 ng/ml somatostatin, 10 ng/ml glycyl-L-histidyl-L-lysine acetate, 5 μg/ml transferrin, 10 nM hydrocortisone, 1×10^{-3} units of TSH and 10 μg/ml insulin (Sigma Chemical Co., St Louis, MO, USA)) (15). Cells were plated at various densities into appropriate tissue culture vessels at least 5 days prior to the start of any experiment. The cultures were incubated at 37˚C in 5% CO_2: 95% air with medium changes approximately every three days. The cells were maintained in medium with penicillin/streptomycin (1%) and routinely tested for Mycoplasma contamination.

Source of radiation. The γ-rays for these studies had energies of 1.17 and 1.33 MeV and linear-energy transfer (LET) of 0.267 KeV/μm. The radiation was delivered by an El Dorado cobalt-60 teletherapy unit (Atomic Energy of Canada, Ltd., Commercial Products Division, Ottawa, Canada). The beam was directed vertically downward to project a 30 x 30 cm field at isocenter 80 cm from the source. The dose rate was approximately 0.7 Gy/min (17).

A range of time points (6-48 h at the 3-Gy dose) and doses (2-5 Gy at 48 h) were used to test whether there was time or dose dependence for TGF-β1 production in early passage cells (P7). In order to assess for changes in TGF-β1 signaling after irradiation and repeated sub-culturing, early passage cells (P7) were exposed to 2 Gy and subsequently growth-expanded up to a late passage (P18). Early (P7 or P8) and late (P18) passages were then assayed for the appropriate endpoints. The controls in these experiments were 0 Gy early passages (P7 or P8).

Cell proliferation and growth rate. The growth rate of FRTL-5 cells was determined using the CellTitre 96 AO aqueous one solution proliferation assay (MTS assay) from Promega (Madison, WI, USA). The method of conducting this assay has been previously described (14). Standard curves were generated from cells plated at known density, in a 96-well plate, (5×10^4 cells/well, which were two-fold serially diluted) to enable normalization for the level of dye reduction to cell number. Experimental cells were established in 96-well plates starting at a density of 5×10^3 cells/well and assayed for their growth rate after 48 h. The absorbance of the formazan product was read at 490 nm wavelength in a Bio-Rad Micro-plate spectrophotometer (Bio-Rad Laboratories, Philadelphia,

PA, USA) and was directly proportional to the number of living cells in the culture. To confirm our results, the cells were counted by two other methods. First, the cells were labeled with propidium iodide (PI) and counted *via* a Laser Scanning Cytometer (LSC) (CompuCyte, Cambridge, MA, USA) (17). Second, the cells were harvested in trypan blue and manual counts were conducted using a hemocytometer.

Enzyme-linked immunosorbent assays (ELISA) for TGF-β1. A commercially available human TGF-β1 ELISA kit (Quantikine™ kit, R & D Systems, Minneapolis, MN, USA), based on binding of TGF-β1 to the soluble TβRII receptor, was used to determine the levels of the cytokine released into supernatants collected from the non-irradiated and irradiated FRTL-5 cell cultures. In order to quantify the total amount of TGF-β1 (latent + active forms) present, the supernatants were treated with 1 N HCl followed by 1.2 N NaOH/0.5 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer to activate the latent TGF-β1 present in the supernatants. The concentration of TGF-β1 in each test sample was calculated from the standard curve obtained by plotting the optical density (OD 450 nm) of the standards against their respective concentrations.

Immunoblotting for TGF-β1 receptor type I and II. FRTL-5 cells, from various passage numbers (irradiated and non-irradiated), were cultured to approximately 80% confluency. The protein lysates were then obtained by scraping in cold immunoprecipitation buffer (IPB; 50 mM Tris-HCl pH 7.4, 1% NP-40, 5% Glycerol, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 2 μg/ml of each protease inhibitor (aprotinin, leupeptin and pepstatin), 2 mM Na3VO4, 2 mM NaF and 1% Triton-X) using 1 ml/100 mm plate. The protein lysates were incubated on ice for 45 min, sonicated for 30 s and then centrifuged for 10 min at 12,000 rpm. Protein concentrations were determined by conducting Bradford assays (Bio-Rad). The samples were aliquoted and stored at –70˚C prior to use. Appropriate samples, controls and the SeeBlue Plus2 Pre-Stained Standard (Invitrogen, Carlsbad, CA, USA) were resolved on 10% Bis-Tris SDS PAGE gels (Invitrogen) for 50 min at 200V. The proteins were transferred onto 0.2 μm nitrocellulose membranes (Bio-Rad), which were stained with Ponseau S and blocked with blocking buffer (5% non-fat milk, Tris Cl pH 8 (10 mM), 5 M NaCl, Tween 20 (0.05%), deionized water). The blots were hybridized with 2 μl/ml TβRI (mouse monoclonal) or 4 μl/ml RII (rabbit polyclonal) primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in blocking buffer for 1 h at room temperature. They were then washed and incubated with 0.25 μl/ml rabbit secondary antibody or 0.5 μl/ml horseradish peroxidase (HRP)-goat anti-mouse in blocking buffer for ½ h at room temperature (Zymed-Invitrogen, Carlsbad, CA, USA). The resulting blots were incubated with chemiluminescence substrate (Alpha Innotech, San Leandro, CA, USA) and subsequently scanned to TIFF files using an Alpha Imager (Alpha Innotech, address). The blots were re-probed with β-actin (mouse monoclonal) primary antibody (Chemicon, Temecula, CA, USA) as a loading control. The signal was corrected for differences in the amount of protein loaded.

Immunoblotting for Smad proteins. Irradiated and non-irradiated P8 and P18 FRTL-5 cells were retrieved from liquid nitrogen and separately plated on 100 mm petri dishes. The cells were cultured to approximately 80% confluence. Half of the dishes for each cell type were treated with recombinant active TGF-β1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (10 ng/ml was experimentally determined as the ideal concentration for triggering the signaling pathway) for 30 min at 37˚C and the other half was left untreated. Protein lysates were obtained from these cells and immunoblotting was conducted as described above. Nitrocellulose membranes were stained with Ponseau S, blocked with blocking buffer and hybridized with the respective primary antibodies specific for Smad2, pSmad2, Smad3, pSmad3 and Smad4 (Abcam Inc., Cambridge, MA, USA). The blots were re-probed with β-actin (mouse monoclonal) primary antibody as a loading control. The signal was then corrected for differences in the amount of protein loaded.

Construction of pGL3-PAI and pGL3-CAL luciferase reporter plasmids. The cyclin A and PAI-1 plasmids were generously provided by Dr. James Kettering and permission was granted for their use by Dr. Xin-Hua Feng and Dr. Joan Massague. The plasmids were originally placed in pGL2 vectors, which have high luciferase background expression. In order to reduce this background expression, cyclin A and the PAI-1 promoters were amplified, by polymerase-chain reaction (PCR), from pCAL2 and p3TP-Lux plasmids respectively, and inserted into the multi-cloning site of a pGL3 basic vector (Promega, Madison, WI, USA). The PAI-1 promoter of 3TP-Lux (3TP) consists of three 12-O-tetradecanoyl-13-acetate (TPA)-response elements (TREs) and a portion of the PAI-1 promoter fused to the adenovirus E4 promoter (8, 18). The primer sequences used to amplify the cyclin A promoter from pCAL2 were: CGGCGGGGTACCGAGCTCCGTGTTAAATAATTTATGCACATT (forward primer) and CCCCCCAAGCTTCACTGCTCCCGGGAGT GGACGGCGGG (reverse primer). The primer sequences used in amplifying the PAI-1 promoter from p3TP-Lux were: GGACC GAGATCTACATAACCCGGGAGGTACCGAGCT (forward primer) and GGACCCAAGCTTTTTACCAACAGTACCGGAAT GCCAA (reverse primer). The underlined sequences represent restriction sites of *KpnI* (forward cyclin A primer), *HindIII* (reverse cyclin A primer), *BglII* (forward PAI-1 primer) and *HindIII* (reverse PAI-1 primer) respectively. The resulting plasmids constructed were named pGL3-CAL and pGL3-PAI

Transient transfection involving pGL3-CAL and pGL3-PAI. The reporter plasmid pGL3-CAL was used to monitor TGF-β1-dependent cyclin A down-regulation and the pGL3-PAI plasmid to assay TGF-β1-induced PAI-1 induction subsequent to radiation exposure. Zero and 2 Gy P8 and P18 FRTL-5 cells were seeded in 24-well cell culture plates at 1×10^5 cells/well and allowed to reach 60-80% confluence. pGL3-basic empty vector, pGL3-CAL and pGL3-PAI were co-transfected with pRL-TK. pRL-TK, a renilla luciferase reporter gene under the control of the thymidine kinase constitutive promoter, was used as an internal control to measure transfection efficiency. FRTL-5 cells, in each well, were co-transfected with 0.4 μg of DNA comprised of the respective test plasmids and empty vector with pRL-TK at a 10:1 ratio. FRTL-5 cells, in each well, were co-transfected with 0.4 μg of DNA comprised of the respective test plasmids and empty vector:pRL-TK at a 10:1 ratio. Transfections were performed using the Effectene reagent (Effectene Transfection Reagent, Qiagen, Valencia, CA, USA). The cells were then incubated with the transfection complexes for 6 h. The transfected cells were allowed to recover in medium supplemented with serum and hormones for 24 h, after which this medium was replaced with serum-free medium containing all the hormone supplements. TGF-β1 (10 ng/ml) was

then added to the respective culture wells and the plates were incubated for 24 h and 48 h for PAI-1 and cyclin A assays, respectively. The cells were lysed and the firefly/renilla luciferase assay was performed using the Dual Luciferase Reporter Assay kit (Promega). Luciferase activities were measured as relative light units (RLU) using a Berthold Microlumat Plus LB96V luminometer (American Laboratory Trading, Groton, CT, USA). The Firefly luciferase activity, which reflected the promoter activity of cyclin A or PAI-1, was normalized to the Renilla luciferase activity to account for transfection efficiency. The transfection results were represented as Firefly:Renilla ratio (F/R) obtained by dividing the firefly RLU by the renilla RLU.

Statistical analysis. Slope analyses of differences in the rate of cell division between un-irradiated and irradiated cells were represented by linear regression. Statistical comparisons of the slopes between irradiated and un-irradiated cells were judged for significance by the Student's t test (Sigma Stat, Version 3.5, Systat Software, Inc., Chicago, IL, USA). "*p*" values less than 0.05 were considered significant. The differences in the levels of TGF-β1 as a function of time and radiation dose were analyzed by the 1-way analysis of variance (ANOVA) test (SigmaStat software, Version 3.5, address or link). The differences in the basal and active Smads along with the differences in cyclin A and PAI promoter activity of irradiated and un-irradiated cells (P8-P18), treated with or without TGF-β1, were also analyzed by ANOVA.

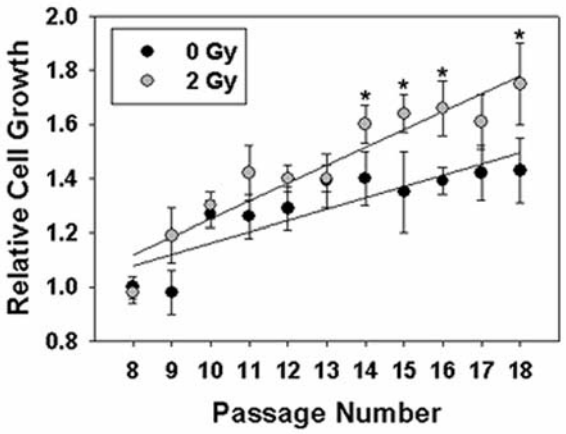

Figure 1. *Cell growth rate of 0 Gy and 2 Gy irradiated FRTL-5 cells as a function of passage number. The MTS OD readings were converted to cell counts and compared to the PI-LSC and manual cell counts. The relationship between relative cell growth and passage number was determined via linear regression (r^2=0.75 for 0 Gy cells and 0.91 for 2 Gy cells). The data, represented as mean±standard deviation (SD), was determined by dividing the normalized cell count of the various passages by 0 Gy pass 8 (P8). The relative cell growth for 0 and 2 Gy cells was then compared using the Student's t test. The growth rate increased from P8-P18 and radiation exposure accelerated this increase. *p<0.05 when 2 Gy is compared to 0 Gy P14-P18.*

Results

Evaluation of changes in FRTL-5 growth rate and TGF-β1 ligand levels after radiation and repeated sub-culturing. In order to measure differences in FRTL-5 cells' rates of growth, non-irradiated and irradiated cells were cultured from early (P8) to late (P18) passages. The relative rate of cell growth increased from P8 to P18 (Figure 1) for both non-irradiated and irradiated cells. The cells exposed to 2 Gy divided at a faster rate than the 0-Gy control cells. Statistically significant differences in the growth rates was noted from P14 to P18 (p<0.05). Data obtained from cells stained with PI and trypan blue confirmed these findings (data not shown).

In order to determine whether defects in TGF-β1 signaling were responsible for the differences observed in growth, some of the major components in the TGF-β1 signaling pathway were examined beginning with the TGF-β1 ligand. The concentration of TGF- β1 was initially measured in the supernatant from irradiated early passage cultures at various times after a 3 Gy-gamma radiation exposure as this dose was previously shown to evoke a robust response (19). The time dependence, using P7 cultures, is illustrated in Figure 2a. These findings revealed that TGF- β1 production increased with time from the 6-h time point to reach a maximum at 48 h after irradiation. The concentration of TGF-β1 then declined at 72 h and increased again slightly after 96 h (Figure 2a). The greatest significance was observed at 48 h compared to all the other time points (p<0.05). Therefore, the maximum TGF-β1 concentration was measured at 48 h post-irradiation for all subsequent experiments.

Next, we determined the dose response for production of TGF-β1 using supernatants from early-passage cultures; the supernatants were harvested 48 h post-exposure. A representative graph from these experiments using P7 cultures is shown in Figure 2b. The results demonstrated that radiation increased the production of TGF-β1 at all doses when compared to the 0-Gy control (p<0.05). The response to radiation was dose-dependent and the highest level of TGF-β1 was obtained at 3 Gy. In fact, statistically significant differences were observed at this dose when compared to the other doses (p<0.05). Although the 3-Gy dose caused the highest production of TGF-β1, we used 2 Gy for subsequent experiments to minimize toxicity while assuring an adequate response.

To investigate whether changes in the production of TGF-β1 occurred as a result of radiation and repeated sub-culturing, early (P8)- and late (P18)-passage cultures were compared. Even though radiation caused a significant increase (p<0.05) in TGF-β1 in early cultures as expected, the levels returned to that of the control (0 Gy, P8) after repeated sub-culturing. There were no significant changes in the TGF-β1 levels of the un-irradiated late passage cells when compared to the control (p>0.05) (Figure 2c).

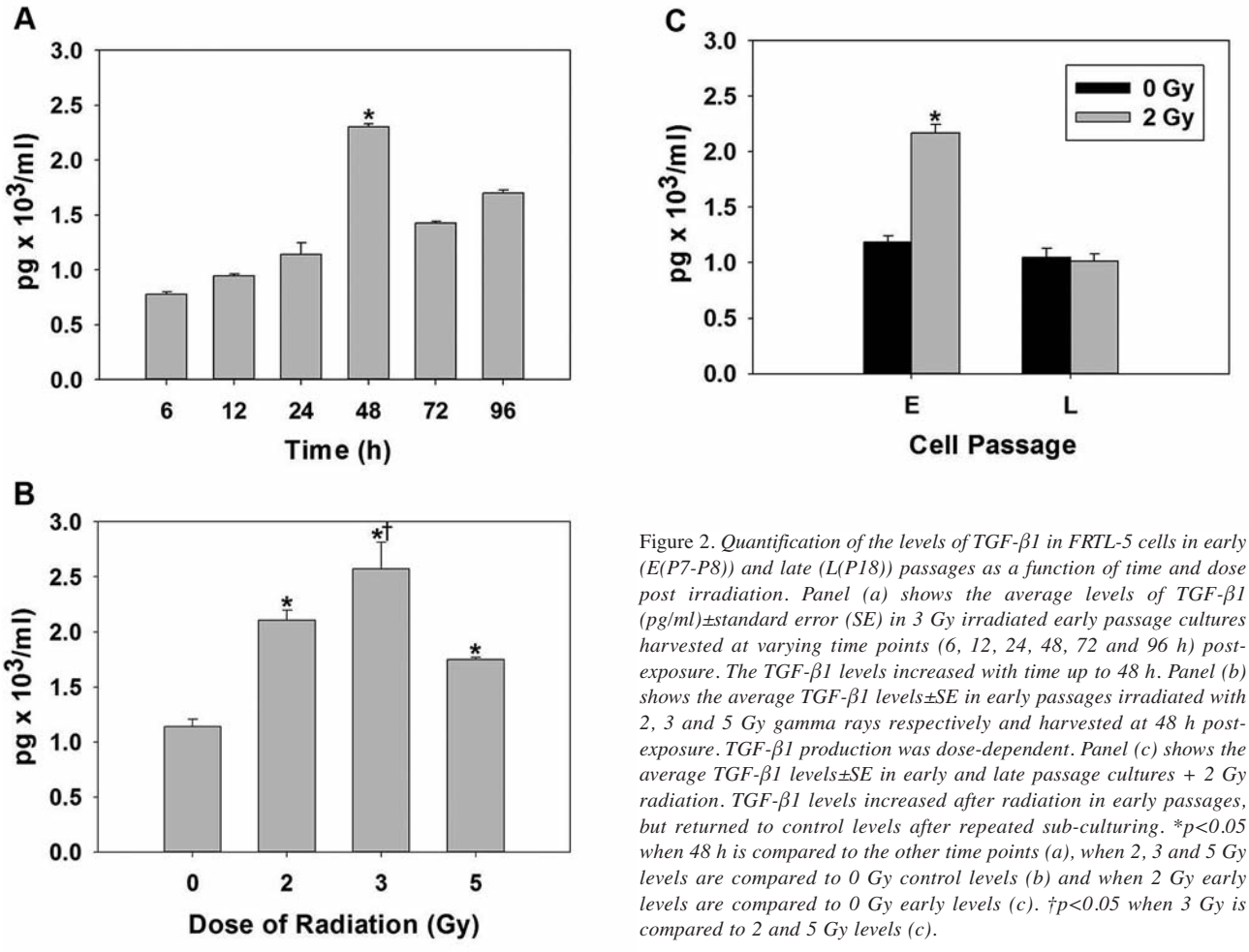

Figure 2. *Quantification of the levels of TGF-β1 in FRTL-5 cells in early (E(P7-P8)) and late (L(P18)) passages as a function of time and dose post irradiation. Panel (a) shows the average levels of TGF-β1 (pg/ml)±standard error (SE) in 3 Gy irradiated early passage cultures harvested at varying time points (6, 12, 24, 48, 72 and 96 h) post-exposure. The TGF-β1 levels increased with time up to 48 h. Panel (b) shows the average TGF-β1 levels±SE in early passages irradiated with 2, 3 and 5 Gy gamma rays respectively and harvested at 48 h post-exposure. TGF-β1 production was dose-dependent. Panel (c) shows the average TGF-β1 levels±SE in early and late passage cultures + 2 Gy radiation. TGF-β1 levels increased after radiation in early passages, but returned to control levels after repeated sub-culturing. *$p<0.05$ when 48 h is compared to the other time points (a), when 2, 3 and 5 Gy levels are compared to 0 Gy control levels (b) and when 2 Gy early levels are compared to 0 Gy early levels (c). †$p<0.05$ when 3 Gy is compared to 2 and 5 Gy levels (c).*

Collectively, these data suggest that ionizing radiation causes an elevation in TGF-β1 production, which is time- and dose-dependent. However, in late-passage cells + radiation, TGF-β1 levels were not significantly different from control cells (0 Gy, P8). Even though repeated sub-culturing had little effect on the TGF-β1 concentration, other phenotypic changes suggested that the cells were no longer responsive to TGF-β1 growth inhibition. Therefore, other factors important in TGF-β1 signaling were examined, *i.e.*, receptors for the cytokine, Smad proteins, cyclin A and PAI-1.

Analysis of TGF-1 receptor expression. Previous studies have reported that equal amounts of the TGF-β1 receptors (TβRI and TβRII) are necessary for normal cellular responses (9, 20). Therefore, we quantified these receptors by western blot analysis and immunocytochemistry. Representative Western blots of TβRI and TβRII in early (P8)- and late (P18)-passages are depicted in Figure 3a and c respectively. Their corresponding densitometry values are shown in Figure 3b and d. Table I lists the relative ratios of TβRI to TβRII. These results indicate that neither radiation exposure nor repeated sub-culturing alters the protein levels or ratios of the TGF-β1 receptors ($p>0.05$). The results of immunocytochemistry (data not shown) were in agreement with the western blot analysis.

Evaluating changes in total and phosphorylated Smad levels after radiation and repeated sub-culturing. Given that there were no changes in TGF-β1 receptor expression due to radiation and/or repeated sub-culturing, the investigation shifted to measuring the next downstream signaling molecules of the TGF-β1 pathway, the Smad proteins. Western blots were performed to determine whether there were changes in the protein levels or in the phosphorylation patterns of the Smad proteins in the FRTL-5 cells after irradiation or repeated sub-culturing, with or without the addition of exogenous TGF-β1. Exogenous TGF- β1 was added to amplify effects that would not have otherwise been readily-detected with endogenous levels only.

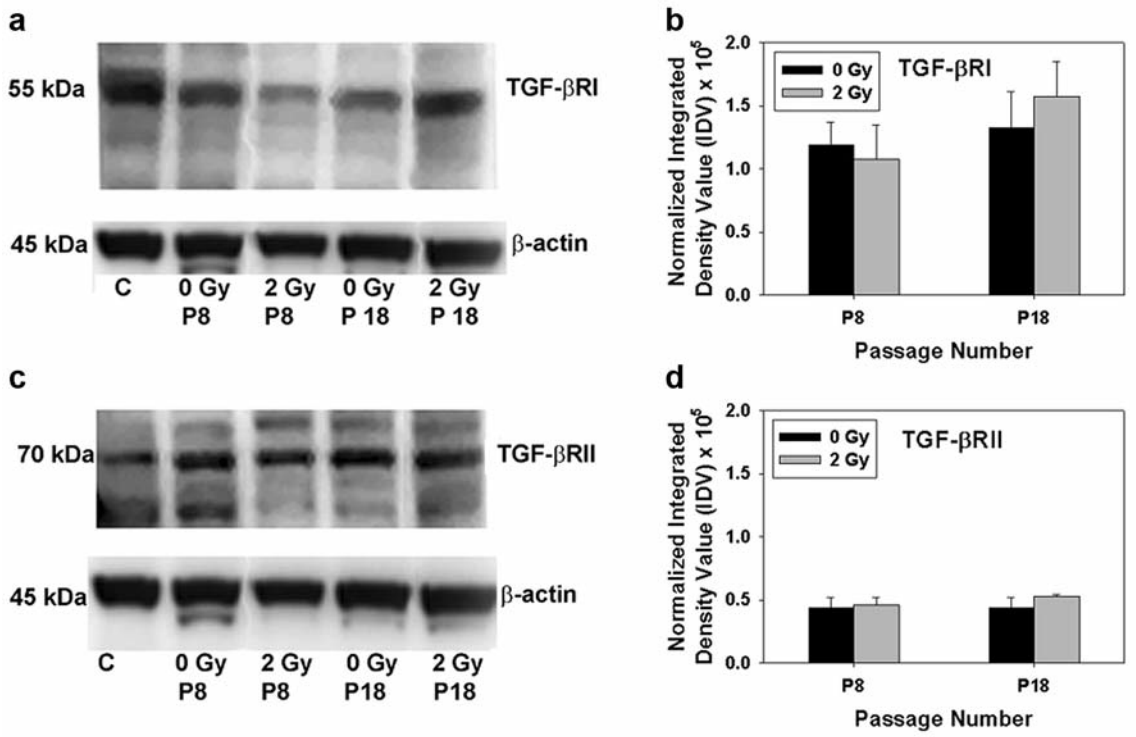

Figure 3. *TGF-β receptor levels as a function of radiation and passage number. Panel (a) shows the representative immunoblots of TβRI (upper blots) and β-actin control (lower blots) in FRTL-5 (P8 and P18) cells ± irradiation. Panel (c) shows the representative immunoblots of TβRII (upper blots) and β-actin control (lower blots) in FRTL-5 (P8 and P18) cells ± radiation. Normalized densitometer values, for TβRI and TβRII, are shown in panels (b) and (d) respectively, which are represented as the mean±SE of three independent experiments. There were no significant changes in the levels of TβRI and TβRII (p>0.05).*

Representative western blots of pSmad2, Smad2 and the β-actin loading control are shown in Figure 4a. After radiation and/or repeated sub-culturing, there was a steady decrease in the levels of Smad2 phosphorylation in cells that were not exposed to exogenous TGF-β1. Actually, pSmad2 levels were significantly low ($p<0.05$) in 2 Gy P8, 0 Gy P18 and 2 Gy P18 cultures when compared to 0 Gy P8 (Figure 4b). When cells were exposed to exogenous TGF-β1, there was a significant increase ($p<0.05$) in the pSmad2 levels when compared to untreated cells in both early- and late-passage cultures ± radiation. However, the pSmad2 levels also decreased in TGF-β1 treated cells that were irradiated and repeatedly sub-cultured. Statistical significance ($p<0.05$) was observed only in 2 Gy P18 cells compared to 0 Gy P8 control cells (Figure 4b). There was a similar decreasing trend in total Smad2 for cells that were repeatedly sub-cultured and/or irradiated independent of exogenous TGF-β1 addition. However, this decrease was not significant ($p>0.05$) (Figure 4c).

Representative western blots of pSmad3, Smad3 and the β-actin loading control are shown in Figure 5a. In cells not treated with TGF- β1, the levels of pSmad3 appeared to slightly decrease after radiation without a further reduction following radiation and/or repeated sub-culturing. However, this change was not statistically significant ($p>0.05$) (Figure 5b). When cells were exposed to exogenous TGF-β1, there

Table I. *Relative ratio of TGF-β1 receptor levels in FRTL-5 cells as a function of radiation and repeated sub-culturing.*

Dose (Gy) and passage (P) #	Mean[a]±S.E.[b]	n[c]
0 Gy P8	1.00±0.00	3
2 Gy P8	0.80±0.19	3
0 Gy P18	1.00±0.00	3
2 Gy P18	1.03±0.28	3

The ratio of TβRI to TβRII was calculated by dividing the corrected integrated densitometer readings (IDV) of TβRI by those of TβRII. From these ratios, the relative ratio TβRI to TβRII was calculated by dividing the ratio of the 2 Gy samples by that of the 0 Gy samples. P8, early passage; P18, late passage. [a]Mean of three independent experiments. [b]Standard error of the mean for three independent experiments. [c]Number of replicates.

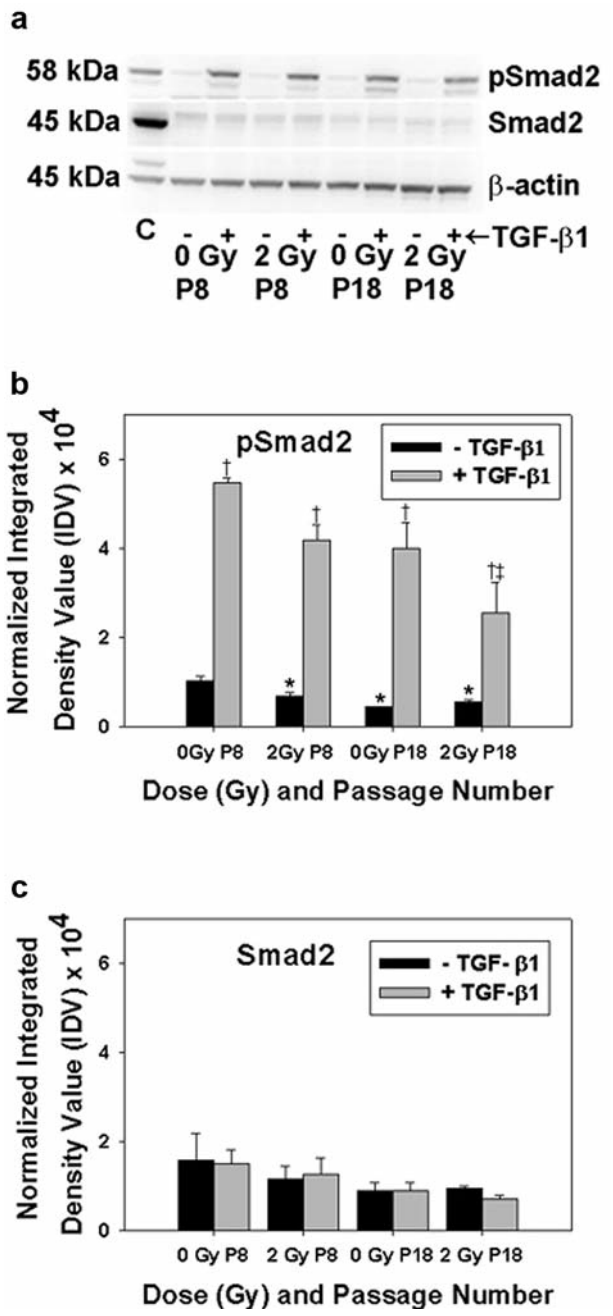

Figure 4. *Total and phosphorylated Smad2 levels as a function of radiation and repeated sub-culturing. Panel (a) shows representative immunoblots of pSmad2 (upper blot), total Smad2 (middle blot) and β-actin control (lower blot) in P8 and P18 FRTL-5 cells ± irradiation. The corresponding normalized densitometric values for pSmad2 and total Smad2 are shown in panels (b) and (c) respectively (mean±SE, n=3). The levels of pSmad2 decreased steadily after radiation and repeated sub-culturing with or without exogenous TGF-β1 compared to control (0 Gy P8) (b). The decreased trend in the total Smad2 levels (c) was not significant (p>0.05). *indicates p<0.05 when 2 Gy P8, 0 Gy P18 and 2 Gy P18 are compared to 0 Gy P8 – TGF-β1. †p<0.05 when all cells treated with exogenous TGF-β1 are compared to cells not treated with TGF-β1. ‡p<0.05 when 2 Gy P18 cells are compared to 0 Gy P8 cells +TGF-β1.*

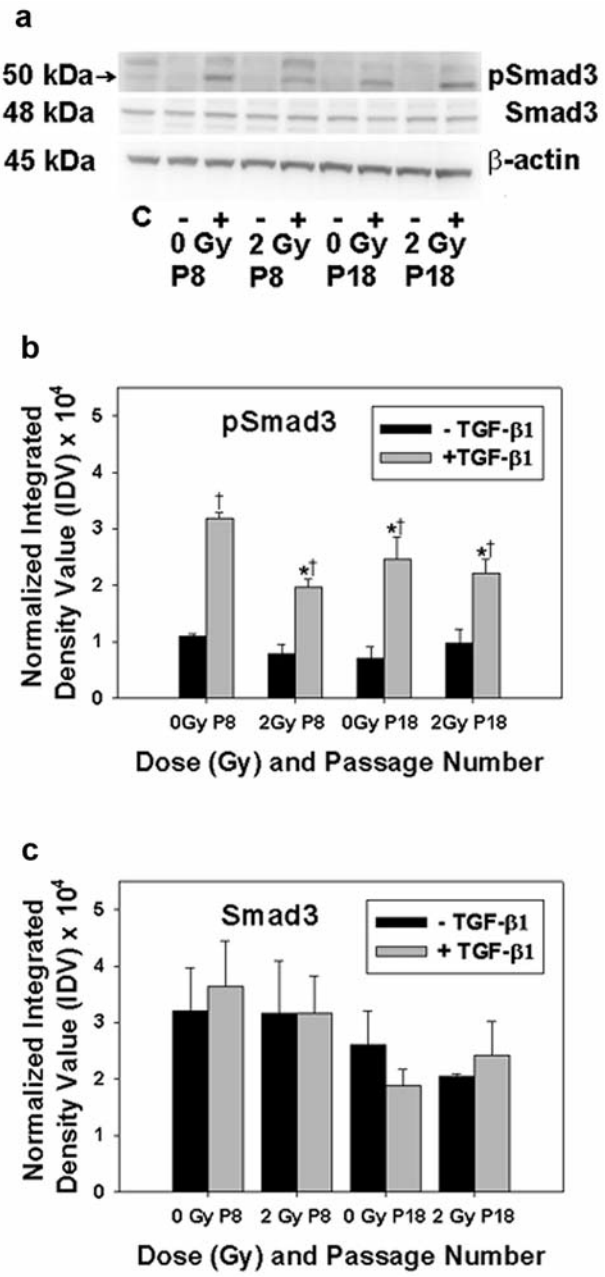

Figure 5. *Total and phosphorylated Smad3 levels as a function of radiation and repeated sub-culturing. Panel (a) shows representative immunoblots of pSmad3 (upper blot), Smad3 (middle blot) and β-actin control (lower blot) in P8 and P18 FRTL-5 cells ± irradiation. The corresponding normalized densitometric values of pSmad3 and Smad3 are shown in panels (b) and (c) respectively (mean±SE, n=3). In TGF-β1 treated cells, the levels of pSmad3 decreased significantly after radiation but did not decrease any further in cells that were repeatedly sub-cultured ± radiation (b). A similar trend was observed in cells not treated with TGF-β1 even though the change was not significant. The levels of total Smad3 did not change significantly under our experimental conditions (p>0.05) (c). †p<0.05 when all cells treated with exogenous TGF-β1 are compared to cells not treated with TGF-β1. *p<0.05 when 2 Gy P8, 0 Gy P18 and 2 Gy P18 are compared to 0 Gy P8 + TGF-β1.*

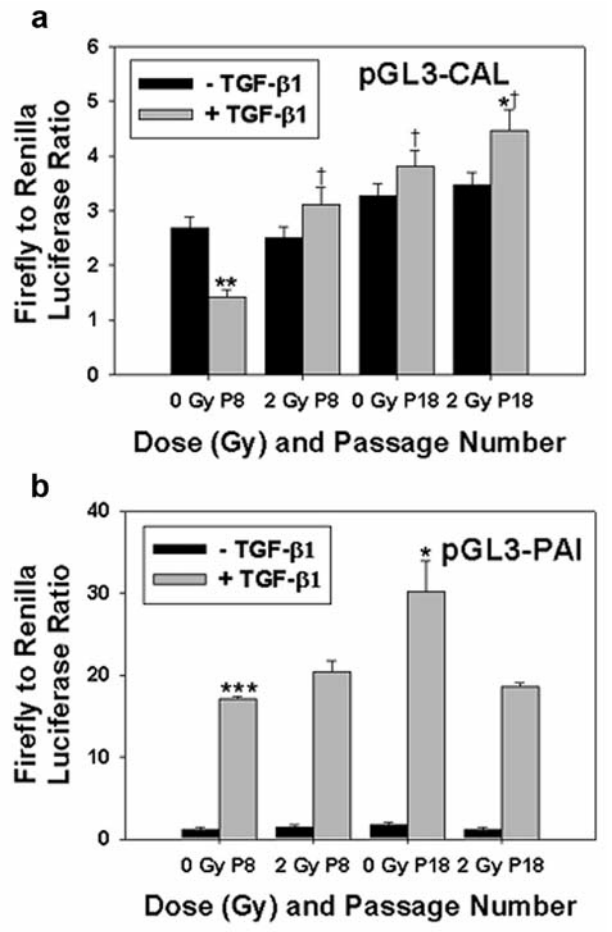

Figure 6. *TGF-β1 regulation of cyclin A and PAI-1 reporter plasmid transcription in irradiated and non-irradiated P8 and P18 FRTL-5 cells. Panel (a) shows the luciferase activity of pGL3-CAL (transcriptional activity of cyclin A) represented by the average firefly:renilla luciferase ratio±standard deviation (SE) for six independent transfections. Significance was observed in all the groups compared to 0 Gy P8 + TGF-β1. Cyclin A activity for 2 Gy P18 + TGF-β1 was also significantly different from that of 2 Gy P18 - TGF-β1. Panel (b) shows the luciferase activity of pGL3-PAI (transcriptional activity of PAI-1) represented by the average firefly:renilla luciferase ratio ± SE for six independent transfections. Significance was observed for 0 Gy P18 + TGF-β1 compared to 0 Gy P8 + TGF-β1. **p<0.005 when 0 Gy P8 cells + TGF-β1 are compared to 0 Gy P8 cells - TGF-β1 (a). †indicates p<0.005 when 2 Gy P8, 0 Gy P18 and 2 Gy P18 are compared to 0 Gy P8 + TGF-β1 cells (a). *p<0.05 when 2 Gy P18 + TGF-β1 is compared to 2 Gy P18 - TGF-β1 (a) and when 0 Gy P18 is compared to 0 Gy P8 + TGF-β1 (b). ***p<0.001 when 0 Gy P8 + TGF-β1 cells are compared to 0 Gy P8 - TGF-β1 cells (b).*

was a significant increase ($p<0.05$) in the pSmad3 levels when compared to untreated cells in both early-and late-passage cultures ± radiation. In TGF-β1-treated cells the decrease in pSmad3 was significant in irradiated (2 Gy P8) cells compared to 0-Gy P8 control cells ($p<0.05$). Repeated sub-culturing ± radiation did not cause the pSmad3 levels to decrease any further than they did with radiation only (0-Gy P18 and 2 Gy P18). Nevertheless, the pSmad3 levels in these cells were also significantly lower ($p<0.05$) than the 0 Gy P8 cells (Figure 5b). There were no significant differences in the total levels of Smad3 in early or late passage numbers, regardless of whether the cells were exposed to γ-rays or treated with exogenous TGF-β1 ($p>0.05$) (Figure 5c). When we examined Smad4 levels, there were no significant changes under our experimental conditions.

Effects of radiation and repeated sub-culturing on TGF-β1 regulation of cyclin A and PAI-1 transcription. TGF-β1 functions to arrest cell-cycle progression by decreasing the production of cyclin A (10, 11, 13, 21). TGF-β1 also functions to increase extracellular matrix (ECM) deposition by increasing the levels of PAI-1 (8, 18). Transcription of these downstream effectors is regulated, in part, by Smads2 and 3, which act as transcriptional factors (18, 22, 23). Since radiation exposure and/or repeated sub-culturing resulted in decreased Smad2 and 3 activities, we sought to determine whether these transcription factor changes altered TGF-β1's ability to correctly regulate cyclin A and PAI-1 transcription.

The pGL3-CAL plasmid was used to determine the effects of radiation and repeated sub-culturing on cyclin A promoter regulation. As expected, in un-irradiated early-passage cells (0 Gy P8), the addition of exogenous TGF-β1 decreased cyclin A promoter activity significantly (indicated by a decrease in luciferase activity) ($p<0.005$) (Figure 6a). However, in cells that were irradiated and repeatedly sub-cultured, TGF-β1 did not trigger a decrease in cyclin A production. In fact the cytokine enhanced cyclin A promoter activity under these conditions, especially in late-passage cells (Figure 6a). This increase in TGF-β1-induced cyclin A promoter activity was significant at 2 Gy P8, 0 Gy P18 and 2 Gy P18 when compared to 0 Gy P8 control cells ($p<0.005$). A significant increase in TGF-β1-induced activity was also measured in 2 Gy P18 + TGF-β1 compared to 2 Gy P18 - TGF-β1 ($p<0.05$). There were, however, no significant changes in cyclin A promoter activity for cells that were not treated with TGF-β1.

The effect of radiation and repeated sub-culturing on the PAI promoter activity was determined using the pGL3-PAI plasmid. The addition of exogenous TGF-β1 caused a significant increase in PAI-1 transcriptional activity, as indicated by an increase in luciferase activity (Figure 6b) in all treatment groups ($p<0.001$). Radiation did not significantly alter TGF-β1's induced activity of PAI-1. However, the increase in luciferase activity induced by TGF-β1 was further enhanced by repeated sub-culturing (0 Gy P18; $p<0.05$ compared to 0 Gy P8). These findings indicate that repeated sub-culturing, but not radiation, played a role in enhancing PAI-1 transcriptional activity (Figure 6b).

Discussion

In one of our previous studies with FRTL-5 cells, we tracked Cx32 gap junction changes as a function of repeated sub-culturing and found an acceleration of growth rate with passage number and the appearance of cells bearing mutation in Cx32. In cultures beyond P18, the loss of intercellular dye transfer due to truncated Cx32 correlated with the increase in cell division; by P25 the doubling time was half that of early-passage cells (14). Increasing growth rate with repeated passage number and an acceleration of the growth rate following radiation exposure were documented in cultures that were still gap junction competent as judged by their ability to transfer microinjected Lucifer yellow dye to contiguous neighboring cells. Thus, factors independent of changes in Cx32 activity must be contributing to the changes in characterized cell phenotype. A likely candidate contributing deregulation of growth was TGF-β1 for which defects in the signaling pathway would result in cells becoming non-responsive to the protein's growth inhibitory effect. An increase in TGF-β1 production was measured in FRTL-5 cultures following radiation, which is consistent with reports by us and others (2, 19, 24). However, the stimulation of TGF-β1 production by radiation was not observed in late cultures. Further observations that the differences in the phenotypes with passage number were not associated with changes in TGF-β1 ligand production, led us to investigate whether downstream components in the TGF-β1 signaling pathway were defective, thus resulting in cells that were no longer responsive to the normal growth inhibitory function of TGF-β1.

The findings of this study indicate that neither radiation nor passage number modified the levels of the TGF-β1 receptors, TβRI and TβRII. Similarly, no effect was observed on the total levels of Smads2, 3 or 4 ± exogenous TGF-β1 under our experimental conditions. However, radiation and/or repeated sub-culturing caused a decrease in the phosphorylation patterns of Smads2 and 3, which may have accounted for a defect in TGF-β1's ability to cause cell growth inhibition, especially in early-passage cells where TGF-β1 levels increased. TGF-β1's inability to correctly regulate cyclin A was observed in cells transfected with pGL3-CAL, which further indicates that the signaling pathway has become defective. It was also observed that repeated sub-culturing, and not radiation, contributed to a greater enhancement of TGF-β1-induced PAI-1 production.

Changes in functional responses to TGF-β1 have been reported to be the result of a loss or unbalanced expression of one of the TGF-β1 receptors, TβRI and TβRII. Studies conducted by various laboratories showed that a change in the levels and/or ratios of TβRI and TβRII are associated with an altered signaling pathway in transformed FRTL-5 cells (9), prostate cancer cells (20) and MCF-7 breast cancer cells (25, 26). Even though our investigation did not confirm these findings, the possibility should not be ruled-out that differences may have occurred in their structure that rendered them non-functional. A study by Reeves *et al*. showed that radiation induced TGF-β1-mediated cell growth inhibition *via* reduced cell-cycle regulation in spontaneously immortalized lung epithelial cells expressing a defective TβRII (Δ RII) compared to primary lung cells that expressed normal TβRII (2). It is therefore possible that radiation and/or repeated sub-culturing could cause TGF-β1 receptors to become structurally defective, thus altering the signaling pathway and making the FRTl-5 cells no longer responsive to TGF-β1.

A similar possibility holds true for the Smad proteins. Although there were no significant differences observed in the basal levels of Smad4, structural alterations may have occurred under our experimental conditions, which may have disrupted its ability to co-localize with pSmads2 and 3 (forming a transcription complex), thus altering the signaling pathway. This theory is supported by studies reporting that the most frequently mutated *Smad* gene is *Smad4* and that mutations of this nature tend to alter the structure of this Smad protein, preventing it from forming effective hetero-oligomerization with *Smads2* and *3* (13, 27, 28). In tumorigenic cells, mutations have also been shown to occur in the MH2 domains of Smads2 and 3, thus preventing them from associating with or becoming phosphorylated by TβRI. As a result, these receptor-regulated Smads (R-Smads) are degraded rapidly and cause alterations in TGF-β1 signaling (13, 23). It is, therefore, possible that similar mechanisms may be operating in our FRTL-5 cells after radiation and/or repeated sub-culturing, which resulted in reduced levels of activated Smads observed in our study and, thus, resulted in the cells becoming non-responsive to TGF-β1's growth inhibition. Closer investigation into the structure of the receptors and Smad proteins are required in order to confirm these theories.

The transcription of cyclin A and PAI-1 (downstream effectors of TGF-β1 signaling) is regulated, in part, by Smads2 and 3, which act as transcriptional factors. Thus, we suspected that a decrease in these Smad activities, as a result of repeated sub-culturing and radiation, altered TGF-β1's ability to correctly regulate cyclin A and PAI-1 transcription. The current findings, regarding cyclin A transcription, confirmed our speculation suggesting that changes in the levels and activities of Smads2 and 3 after repeated sub-culturing and radiation resulted in TGF-β1's inability to correctly regulate cyclin A production and, thus, caused cells to be non-responsive to growth inhibition by TGF-β1. The results also demonstrated that γ-radiation accelerated the growth rate of FRTL-5 cells. The addition of exogenous TGF-β1 resulted in increased promoter activity compared to TGF-β1-untreated cells that were irradiated and repeatedly sub-cultured. This suggests that, under our experimental conditions, TGF-β1 may be cross-talking with other pathways [such as the mitogen-activated protein kinase (MAPK) pathways] to induce cyclin A

production instead of functioning normally to inhibit this cell-cycle promoter. It would, therefore, be important to examine the players in these pathways to determine whether they influence cell-cycle progression in irradiated and repeatedly sub-cultured FRTL-5 cells. Investigation of molecules regulated by TGF-β1, such as other cyclins/cyclin-dependent kinases, cyclin-dependent kinase inhibitors, other tumor suppressors (*e.g.* Rb) and oncogenes (*e.g.* c-Myc) in future studies will further confirm whether TGF-β1's function was repressed or switched to that of a growth promoter following repeated sub-culturing and radiation.

On the other hand, a defect in the TGF-β1 signaling pathway did not result, as expected, in a decrease in *PAI* promoter activation. Moreover, it caused a further induction in PAI transcription as a result of repeated sub-culturing, but not radiation. We also examined the PAI-1 protein levels in FRTL-5 cell lysates and supernatants. The results confirmed that late passage cells exhibited an enhanced TGF-β1 dependent PAI-1 production *versus* early passage cells and that radiation effects were minimal (data not shown). Radiation-induced fibrosis is an important side effect of radiation therapy for cancer. One of the molecules implicated in radiation-induced fibrosis is PAI-1 (29) whose normal function is to enhance ECM accumulation by inhibiting urokinase plasminogen activator (u-PA) or tissue-type plasminogen activator (t-PA) (30, 31). Studies have shown that radiation caused an increase in the *PAI-1* mRNA levels 24, 32). This increase was found to be dose-dependent (32). Hageman *et al*. also demonstrated that PAI-1 levels were dose-dependent in Mv1Lu cells irradiated with varying doses of gamma radiation. This trend was observed with or without exogenous TGF-β1 (29). The results, obtained at the 2-Gy dose in our study, were similar to the results they obtained in that, at this dose, radiation did not enhance PAI-1 induction either with or without exogenous TGF-β1. Significant effects of radiation were only observed at doses from 8-32 Gy. They also determined that when four fractionated doses of 2 Gy were administered over a four-day period there were significant radiation-induced increases in PAI-1 activation. This was further enhanced significantly when TGF-β1 was administered along with the fractionated doses. Therefore, a 2-Gy dose of gamma irradiation has implications in radiation-induced fibrosis when it is administered in fractions (reaching a much higher total dose) instead of as a single dose.

Our results show that repeated sub-culturing results in a further induction in PAI-1 at the transcription and protein levels and is consistent with reports of other investigators, which suggest that increased PAI-1 production is implicated in cell transformation and tumor metastasis (30, 33, 34). Other experiments concerning molecules involved in ECM accumulation and degradation would greatly aid in determining whether repeated sub-culturing and radiation modify ECM turnover and whether this effect is enhanced by TGF-β1. Insight would, thus, be gained as to whether these molecules assist in the development of a tumorigenic phenotype.

Overall, the presented data suggest that defects in TGF-β1 signaling contribute to the alterations measured in FRTL-5 cells as a result of radiation and repeated sub-culturing. To date, this study is one of the first to give partial insight into the mechanism by which FRTL-5 cells respond to the effects of radiation and repeated sub-culturing. Clearly, additional studies are warranted to advance the field and further determine the mechanism(s) that affect TGF-β1 signaling in FRTL-5 cells. This cell line has proven to be a useful model to study radiation-induced diseases and processes involved in resistance to TGF-β1's control of cell growth, thus assuming a potentially malignant phenotype. Increased knowledge in this area is important in order to facilitate development of new strategies that further improve the outcome of radiotherapy.

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