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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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 (60Co) 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 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 were sub-cultured two-fold serially diluted) to enable normalization for the level of proliferation assay (MTS assay) from Promega (Madison, WI, USA). 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, (5x10^4 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 5x10^3 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, USA).
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 Ponceau 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 (TFA)-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: CGGCCGTTACGAGCTCCGTGGTTAATATTTATGCACTATT (forward primer) and CCCCCAAGCTTACGCTTCCCCGGAGTGAGGACCGCGGG (reverse primer). The primer sequences used in amplifying the PAI-1 promoter from p3TP-Lux were: GGACCAGATCTACATAACCCGGGGAGGTACGGAGCT (forward primer) and GGACCCAGCTTTTACCAACAGTACGGGATGCCAA (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 1×10⁵ 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
increased again slightly after 96 h (Figure 2a). The greatest concentration of TGF-β1 then declined at 72 h and time point to reach a maximum at 48 h after irradiation. The time dependence, using P7 exposures as this dose was previously shown to evoke a robust response (19). The differences in the level 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 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.

**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 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 (r²=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.](image-url)
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.
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

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

<table>
<thead>
<tr>
<th>Dose (Gy) and passage (P)</th>
<th>Mean±S.E.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy P8</td>
<td>1.00±0.00</td>
<td>3</td>
</tr>
<tr>
<td>2 Gy P8</td>
<td>0.80±0.19</td>
<td>3</td>
</tr>
<tr>
<td>0 Gy P18</td>
<td>1.04±0.00</td>
<td>3</td>
</tr>
<tr>
<td>2 Gy P18</td>
<td>1.03±0.28</td>
<td>3</td>
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</table>

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.
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 (A 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 oncopogenes (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. However, 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 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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