TGF beta-1 dependent fast stimulation of ATM and p53 phosphorylation following exposure to ionizing radiation does not involve TGF beta-receptor I signalling

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TGFβ-1 dependent fast stimulation of ATM and p53 phosphorylation following exposure to ionizing radiation does not involve TGFβ-receptor I signalling

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Abstract

***Background and purpose*: It has been proposed that radiation induced stimulation of ATM and downstream components involves activation of TGFβ-1 and that this may be due to TGFβ-1-receptor I-Smad signalling. Therefore, the aim of this study was to clarify the distinct role of TGFβ-1-receptor I-Smad signalling in mediating ATM activity following radiation exposure.**

***Materials and methods*: A549 cells were stably transfected with a conditionally regulatable TGFβ-1 antisense construct (Tet-on-system) to test clonogenic activity following irradiation. Phosphorylation profile of ATM, p53, and chk2 was determined in non-cycling, serum-starved cells by immunoblotting. Likewise, A549 wild type cells were used to identify cell cycle distribution as a function of irradiation with or without pretreatment with CMK, a specific inhibitor of furin protease involved in activation of latent TGFβ-1. Furthermore Western and immunoblot analyses were performed on serum-starved cells to investigate the dependence of ATM- and p53-stimulation on TGFβ-1-receptor I-Smad signalling by applying a specific TGFβ-1-receptor I inhibitor.**

***Results*: Knock down of TGFβ-1 by an antisense construct significantly increased clonogenic cell survival following exposure to ionizing radiation. Likewise, CMK treatment diminished the radiation induced G1 arrest of A549 cells. Moreover, both TGFβ-1-knock down as well as CMK treatment inhibited the fast post-radiation phosphorylation of ATM, p53, and chk2. However, as shown by the use of a specific inhibitor TGFβ-1-receptor I-Smad signalling was not involved in this fast activation of ATM and p53.**

***Conclusions*: We confirm that TGFβ-1 plays a critical role in the stimulation of ATM- and p53 signalling in irradiated cells. However, this fast stimulation seems not to be dependent on activation of TGFβ-1-receptor I-Smad signalling as recently proposed.**

Keywords: TGFβ-1; TGFβ-1 receptor signalling; ATM activity

TGFβ-1 is a cytokine with diverse biological activities and has potent, often opposite, effects on various cell types or tissues, e.g., concerning the regulation of cell proliferations and differentiation or cell death as well as extracellular matrix metabolism [7,28]. Moreover, TGFβ-1 has been described to play a key role in the induction and manifestation of radiation induced fibrosis [5,16,19,26,31–33].

TGFβ-1 is secreted as a latent precursor molecule (LTGFβ) which, in turn, is covalently bound to a protein called LTGFβ-binding protein, LTBP [29]. LTBP supports binding of LTGFβ to extracellular matrix molecules and enables proteolytic activation of LTGFβ [6,29]. LTGFβ can be activated by ionizing irradiation through accumulation of reactive oxygen species in the extracellular space of irradiated tissue as well as proteolytic processes [2,10,23]. Once activated, TGFβ-1 is able to bind and activate the TGFβ-receptor-type II (TβRII) dimer which forms a heterotetramer with the TGFβ-receptor-type I (TβRI) dimer. The activated TβRI serine/threonine kinase phosphorylates downstream proteins Smad2/3, which form a complex with Smad4 [18], which then translocates to the nucleus. In concert with specific transcription factors it regulates different genes, e.g., regulating cell cycle control [8,27] or collagen metabolism [12].

In addition, it has been shown that TGFβ-1 plays a role in the cellular response to DNA damage. Radiation induced

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apoptosis in mouse embryonic mammary epithelium appeared to be dependent on TGFβ-1 [11]. In this study depletion of TGFβ-1 either by neutralization or gene knock-out resulted in a decreased p53-Ser-18 phosphorylation in irradiated mouse mammary epithelium. Another connection between p53 and TGFβ-1 was observed by Hageman et al. [15]. An induction of the pro-fibrotic gene PAI-1 occurred after irradiation, which increased strongly after addition of TGFβ-1. This expression was absent after selectively mutating a p53 binding element in the PAI-1 promotor, or in a p53 null cell line. More recently, it was demonstrated that TGFβ-1 modulates the activation of the Ataxia Telangiectasia Mutated (ATM) protein kinase. Following ionizing radiation ATM is rapidly induced via autophosphorylation at Ser-1981 and regulates cell cycle and DNA repair pathways [21]. In a study by Kirshner et al. [22], a marked reduction of ATM activity and downstream effector molecules, like p53, Rad17 and chk2, was observed in mouse TGFβ-1 null keratinocyte. These effects were observed from 15 to 240 min after ionizing radiation. Interestingly, phosphorylation was restored if cells were treated with TGFβ-1 prior to irradiation. Similar results were obtained in human epithelial cells (MCF10A) in which phosphorylation of ATM, p53, chk2 and Rad17 was significantly reduced at 60 min post-irradiation if cells had been treated with TGFβ-1-receptor I-inhibitor (TβR1-inhibitor) prior to radiation exposure [22].

These data indicate that TGFβ-1 may be essential for regulating radiation induced cellular processes which lead to DNA repair and cell cycle control in response to genotoxic stress. Kirshner et al. [22] propose that this regulation is based on extracellular activation of TGFβ-1 and subsequent receptor dependent Smad signalling. However, as shown in the present report inhibition of TβRI-signalling only affected ATM and p53 phosphorylation not earlier than 60 min post-IR. Likewise, as shown for human fibroblast [14] phosphorylation of Smad-2/3 following radiation exposure or TGFβ-1 treatment could also not be observed earlier than 60 min after radiation exposure. Therefore, the question was addressed whether the fast activation of ATM and p53 is solely due to TGFβ-1-receptor dependent Smad-signalling stimulated by extracellular activated TGFβ-1.

Materials and methods

Cell culture

The study was performed using a human bronchial carcinoma cell line A549 (ATCC, USA). Cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (BioWhittaker, USA) and standard amounts of antibiotics (penicillin 100 U/ml; streptomycin 100 μg/ml). With the exception of cell cycle analyses, 72 h serum-starved cells were used for all experimental conditions applied.

TGFβ-1 antisense A549 subclones

A549 cells were stably transfected with the pTETon- and the empty pTRE2-Hygro- or the pTRE2-Hygro-TGFβ-1-antisense vector, respectively, according to the manufacturer's protocol (BD Clontech, Germany) to established a conditionally inducible syngenic system of A549-cells with respect to expression of TGFβ-1. The pTRE2-Hygro-TGFβ-1-antisense vector contained the sequence of the whole human TGFβ-1 reading frame placed in a reverse orientation. Western blot analyses for ATM-Ser1981, p53-Ser15, chk2-Thr68 were done with both, control- and TGFβ-1-antisense vector transfected cells, as described below (see sections Irradiation and FACS analysis) with the exception that cells were treated 48 h before irradiation with doxycycline (1 μg/ml) to induce the TGFβ-1 antisense expression vector.

CMK and Tβ1R-I-inhibitor treatment

CMK is a synthetic furin inhibitor (decanoyl-Arg-Val-Lys-Arg-chloromethyl-ketone) and is a reported tool to inhibit TGFβ-1 activation from LTGFβ-1 [34]. CMK was prepared as a 50 mM stock solution in DMSO. TβRI-Kinase Inhibitor (2 mg/ml; Calbiochem #616451, Germany) was prepared as stock solutions in serum-free culture medium. Working solution of CMK (50 μM) and TβRI-inhibitor (400 nM) was obtained by dilution of stock aliquots with culture medium and was freshly prepared for each experiment. CMK was added to the medium 4 h and TβRI-inhibitor 1 h before irradiation.

Irradiation

All cells were irradiated at room temperature with X-rays (200 kV, 15 mA, filtered by 0.5 mm Cu) generated by a Gulmay RS225 (Gulmay Medical Ltd., GB, dose rate 1 Gy/min) in the dose range of 0—8 Gy.

Colony formation assay

A549 wild type and A549 cells transfected with TGFβ-1-antisense construct were treated with or without doxycycline (1 μg/ml) 48 h before seeding into 6-well plates. Irradiated or unirradiated cells were seeded at a density of 26 cells/cm^2 in 6-well tissue culture plates (9.6 cm^2, Falcon, Becton Dickinson, USA) and incubated for 12 days. The medium of all cultures was renewed every 3 days. Clonogenic cell survival was analysed on the basis of colonies formed from single cell cultures as reported elsewhere [3].

Western blot analysis

Cell lysates were prepared for SDS—PAGE on 10% and 6% (for ATM) resolving gels [9]. Antibodies for detection of the different proteins were diluted in 3% BSA or 3% dry milk in TBS as follows: ATM-pSer-1981 (monoclonal mouse antibody, 1:1000; Upstate/Biomol, Germany), p53-pSer-15 (1:1.000), chk2-pThr-68 (1:1.000), cdc25C-pSer-216 (1:750) (polyclonal rabbit antibodies, Cell Signalling Technology, Inc., USA), p53 (monoclonal mouse antibody, 1:1.000, Oncogene EMD Biosciences, Inc., USA), ATM (monoclonal mouse antibody, 1:1000) and β-actin (polyclonal rabbit antibody, 1:1.000) (Sigma—Aldrich, USA). Protein bands were visualized by using the ECL system (Roche Diagnostics, Germany). For densitometric determination of protein band intensities the ImageJ 1.30 program was applied.

FACS analysis

DNA of A549 cells in the logarithmic growing phase was labelled with 10 μM bromodeoxyuridine (BrdU, Becton Dick-

inson Immunocytometry Systems, USA) for 1 h. Subsequently labelling medium was removed and cells were washed twice in medium to remove non-incorporated BrdU. Cells were then either irradiated with a single dose of 4 Gy or sham irradiated. CMK was given to the medium three times: 2 h before and 12 h as well as 36 h after irradiation (IR). Cell cycle distribution was determined using a FACSort system as described elsewhere [30].

Results

Clonogenic survival

As demonstrated in Fig. 1a, radiation sensitivity of A549 wild type cells was not influenced by treatment with doxycyclin. In contrast doxycycline induction (dox^+) of the anti-TGFβ-1 construct resulted in a significantly better clonogenic cell survival as compared to the not induced condition (without doxycycline, dox^-), i.e., survival fraction of dox^- cells at 3 Gy (SF3) was 0.4025 ± 0.0204 whereas SF3 in dox^+ cells was 0.5710 ± 0.0246 (Fig. 1b).

Flow cytometry

Cell cycle analysis of A549 cells untreated or treated with the furin inhibitor CMK revealed no effect of CMK on cell cycle distribution (Fig. 2). The fraction of BrdU negative cells arrested in G1 within the first 6 h after irradiation alone or in combination with CMK treatment was only 30–46% (Fig. 2a). The proportion of BrdU positive G1-phase cells, i.e., cells which have passed through S-phase once after radiation exposure, was about 20% for both conditions (Fig. 2b). After 6–96 h the fraction of BrdU negative G1-arrested cells decreased from 30% to 12% after irradiation plus CMK treatment and was reduced from 35% to 22% for only irradiated cells (Fig. 2a). Thus, under both conditions the difference in the fraction of BrdU negative G1-cells was in the range of 10–13.2%. Within the same time period after combined irradiation and CMK treatment the fraction of BrdU positive G1-phase cells increased to about 70% whereas in only irradiated cells this fraction was slightly enhanced from 52% to 58% (Fig. 2b). Thus, A549 cells displayed a TGFβ-1 dependent cell cycle arrest after ionizing irradiation which can partly be abolished by concomitant treatment with the furin inhibitor CMK.

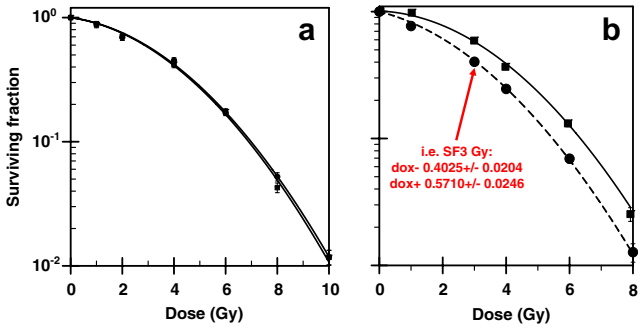

Fig. 1. Clonogenic cell survival of A549 TGFβ-antisense cells following irradiation. (a) Wild type A549 cells treated with (line with squares) or without (line with dots) doxycycline to see an unspecific effect of doxycycline treatment on clonogenic survival. (b) A549 TGFβ-1-antisense clone treated with (line with squares; antisense-TGFβ-1 turned-on) or without (line with dots; antisense-TGFβ-1 turned-off) doxycycline. Data shown represent means ± SE of three independent experiments run in triplicate. In (b) the data points for A549 dox^+ and dox^- cells show significant statistical differences at all radiation doses ($p < 0.05$; $n = 9$).

Time kinetics of radiation induced ATM-activation and downstream pathways

Phosphorylation of ATM at Ser1981 occurred immediately after irradiation with a maximum after 2 min and persisted up to 60 min thereafter. Blocking transcription of TGFβ-1 in dox^+ cells decreased ATM-phosphorylation markedly between 85% (2 min post-IR) and 13% (60 min post-IR) at all time points measured post-irradiation (Fig. 3). Similar results were observed for phosphorylation of p53 at Ser15 (maximum inhibition of 79% 1 min after IR) and phosphorylation of chk2 at Thr68 (maximum inhibition of 76% 30 min after IR) in dox^+ cells (Fig. 3). Corresponding results for ATM and p53 phosphorylation were obtained when A549 wild type cells were treated with CMK before irradiation (data not shown).

Since the cell cycle control proteins chk2 (checkpoint kinase 2) and cdc25C (cell division cycle 25) are effector molecules downstream of ATM kinase we determined the phosphorylation of these proteins (i.e., chk2-Thr68 and cdc25C-Ser216) in A549 wild type cells. As indicated in Fig. 4, a radiation-dependent phosphorylation of chk2, which was sensitive to CMK pretreatment, could be demonstrated. Likewise, irradiation resulted in a fast and strong phosphorylation of cdc25C, which was effectively reduced below control levels after CMK treatment (Fig. 4).

Time kinetics of radiation induced Smad2 phosphorylation

After binding of active TGFβ-1 to the receptor, the activated TβRI kinase initiates Smad signalling through phosphorylation of the Smad2 and Smad3 proteins. Therefore, time kinetics of radiation induced Smad2 phosphorylation were performed to determine the time needed between extracellular TGFβ-1 activation and nuclear translocation of phosphorylated Smad proteins. As shown in Fig. 5 the radiation-dependent fraction of phosphorylated Smad2 is only increased after about 60 min post-IR. As treatment of cells with the TβRI-inhibitor completely abolished phosphorylated Smad2 (Fig. 5) we investigated in subsequent experiments the influence of TβRI-inhibitor on radiation and TGFβ-1 dependent activation of ATM and its downstream signalling.

Influence of TβRI-inhibition on radiation-dependent ATM-signalling

In wild type A549 cells a strong induction of phosphorylated ATM (at Ser1981) and p53 (at Ser15) was apparent already 15 min post-IR. For both proteins, CMK pretreatment resulted in an appr. 60% reduction of the phosphorylation signal (Fig. 6), whereas blocking TβRI by use of the TβRI-inhibitor reduced ATM phosphorylation only by about 30% (Fig. 6). Combined CMK and TβRI-inhibitor treatment

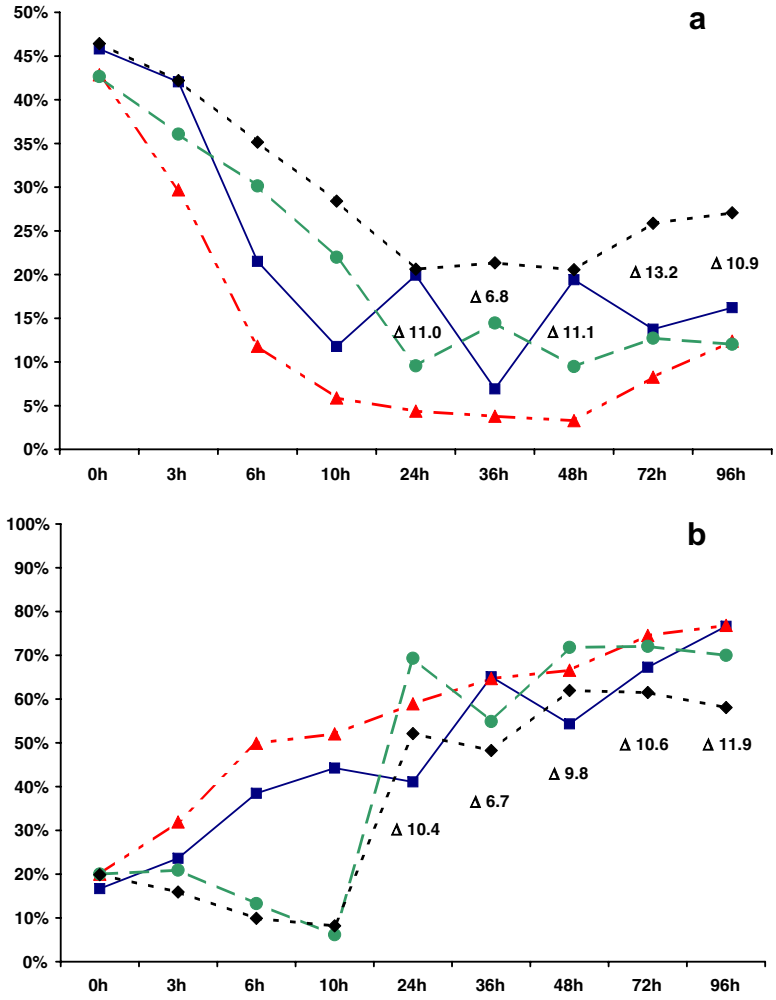

Fig. 2. Cell cycle distribution of wild type A549 cells is TGFβ-1 dependent. (a) Time course of BrdU negative G1 phase A549 cells over a time period of 96 h. (b) Time course of BrdU positive G1 phase A549 cells over a time period of 96 h. Untreated controls (squares, solid blue line) and CMK treated controls (triangle, dash-dotted red line) as well as irradiated cells (diamond, dotted black line) and irradiated CMK treated cells (circle, dashed green line) went through the cell cycle as indicated. Irradiated cells displayed a higher fraction of G1 arrested cells than irradiated and CMK treated cells. The numbers shown represent the difference (Δ) in the fraction of G1-arrested cells between irradiated and irradiated as well as CMK treated cells.

resulted in only a minimal additional effect as compared to CMK treatment alone (reduction about 65%, Fig. 6). Under the same conditions a similar but stronger effect was observed for p53 phosphorylation at ser15. No reduction was apparent after blocking the TβRI, whereas CMK pretreatment as well as CMK treatment combined with TβRI inhibitor reduced p53 phosphorylation by about 40%. Interestingly however, phosphorylation of Smad2 was blocked already 15 min after TβRI-inhibitor treatment indicating a strong inhibition of the receptor function. In contrast CMK pretreatment did not modify effectively Smad2 phosphorylation at the same time point post-IR (Fig. 6).

Discussion

TGFβ-1 is an important tumour suppressor [8] and a potent inducer of growth inhibition and differentiation in several cell types i.e., epithelial cells and fibroblasts [5,16,19,26,28] and plays an important role in the induction and manifestations of radiation induced fibrosis [1,4,17,25]. As shown for normal fibroblasts and several tumour cell lines ionizing radiation effectively induces expression and activation of TGFβ-1 [2,16,23,33], suggesting a role for TGFβ-1 in the regulation of cellular radiation responses, i.e., clonogenic survival as well as cell cycle arrest. This is supported by data of the presented study indicating that clonogenic cell survival following radiation exposure is significantly different in syngenic A549 cells with or without knock down of TGFβ-1 expression. As already reported earlier for homozygous TGFβ-1 knock-out mouse fibroblasts [33] knock down of TGFβ-1 expression by antisense construct leads to enhanced clonogenic cell survival of A549 dox$^+$ cells. Likewise, the furin inhibitor CMK by blocking proteolytic activation of LTGFβ-1 to active TGFβ-1 mediated a decrease in radiation induced G1-arrest in A549 cells. To our knowledge this is the first re-

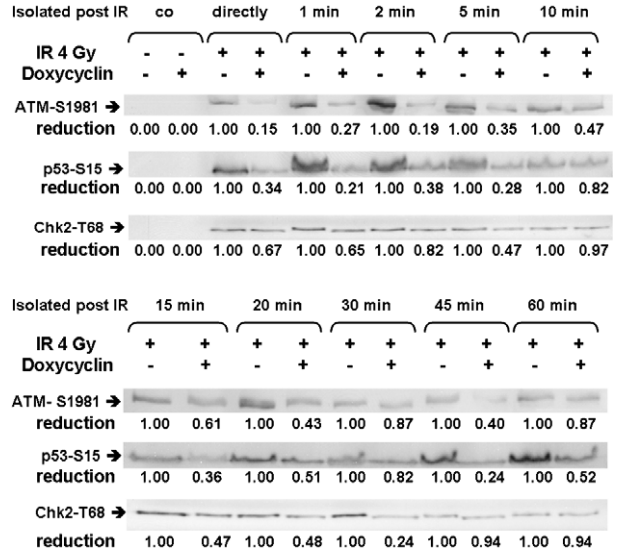

Fig. 3. Radiation induced ATM phosphorylation and downstream signalling are reduced in TGFβ-1 antisense A549 subclones. After switching on the TGFβ-1 antisense (plus doxycycline treatment, dox$^+$), a reduction of ATM and p53 phosphorylation as well as chk2-Thr68 phosphorylation occurred directly after irradiation for up to 60 min in comparison to cells where TGFβ-1-antisense was turned off (dox$^-$). Numbers indicate the densitometric values normalized to 1.0 in dox$^-$ cells for each time point shown.

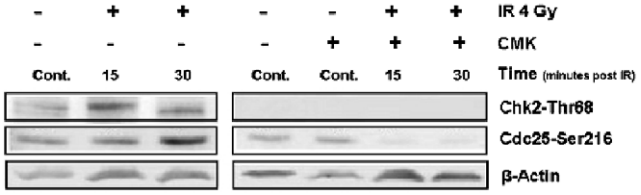

Fig. 4. Radiation induced activation of ATM downstream signals chk2-Thr68 and cdc25C-Ser216 in A549 wild type cells is inhibited by CMK. Phosphorylation of ATM downstream signal components chk2-Thr68 and cdc25C-Ser216 was analysed in A549 wild type cells after irradiation alone (4 Gy), or after pretreatment with CMK and subsequent irradiation (4 Gy). Equal loading was controlled by detecting β-actin.

Fig. 5. Radiation induced Smad2 phosphorylation as a function of TβRI-inhibitor treatment. Lysates of A549 wild type cells irradiated in the presence or absence of TβRI-inhibitor were analysed for the phosphorylation of Smad2 protein. Numbers shown represent the x-fold induction of phosphorylation based on the Smad2 total protein amount of the non-irradiated control.

port that inhibition of proteolytic activation of TGFβ-1 influences both, clonogenic cell survival as well as cell cycle distribution, following exposure to ionizing radiation.

The important role of TGFβ-1 in regulating cellular radiation responses is further supported by Ewan et al. [11] and

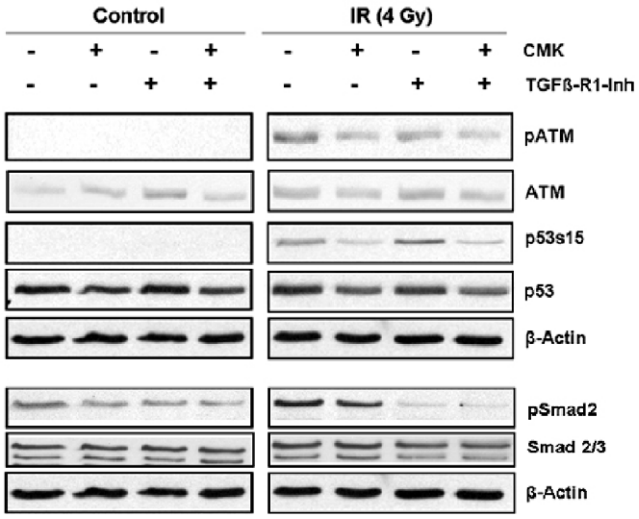

Fig. 6. Radiation induced ATM, p53, and Smad2 phosphorylation as a function of CMK or TβRI-inhibitor treatment. Phosphorylation of ATM, p53, and Smad2 in A549 wild type cells was analysed 15 min after irradiation (4 Gy) with or without pretreatment with CMK (50 μM) or TGFβR1-inhibitor (400 nM) alone as well as in combination of both. Equal loading was controlled by detecting β-actin. Densitometry data were used to calculated the percent differences in ATM and p53 phosphorylation on the basis of actin expression (irradiated controls vs. test conditions).

Hageman [15] who demonstrated a connection between TGFβ-1 and p53 [22]. Other data also show that TGFβ-1 is involved in DNA repair mechanisms by modulating Rad51 expression in epithelial cells [20]. Furthermore, Glick et al. [13] were able to show that TGFβ-1 knock-out keratinocytes lack the typical PALA (*N*-phosphonacetyl-L-aspartic acid, also a DNA-damaging agent) induced p53-dependent G1 arrest. More recently, it was demonstrated by the group of Barcellos-Hoff that ATM kinase, located upstream of p53, may be the first main target of TGFβ-1 signalling in response to ionizing radiation. The authors propose that extracellular TGFβ-1 could be a mediator of intracellular responses to DNA damage [22] through modifying ATM cascade which is important in regulating cellular responses to ionizing irradiation [24,35].

The data presented in our study add further substantial information to the interaction and time kinetics of TGFβ-1 mediated stimulation of ATM and its downstream cascades in irradiated cells. By the use of the protease inhibitor CMK which blocks the protease furin needed for both, intra- and extracellular activation of LTGFβ-1 [9,23] we showed that radiation-dependent phosphorylation of ATM-Ser1981, p53-Ser15, chk2-Thr68 and cdc25-Ser216 could be reduced or completely blocked. However, blocking TGFβ-1 mediated Smad signalling by inhibiting TβRI did not result in these effects. At the first sight this result seems to be in contrast to the data presented by Kirshner et al. [22]. However, the time kinetics of radiation induced ATM- and Smad-phosphorylation presented by Kirshner et al. [22] do not fully support their assumption of a stimulation of ATM-phosphorylation through Smad signalling. ATM-phosphorylation was demonstrated already 15 min post-IR, whereas data for TβRI dependent Smad-phosphorylation were only presented at

60 min post-IR. This difference in time kinetics is supported by our present and previous report [14]. Therefore, we tested whether inhibition of TβRI in irradiated cells prevents ATM- as well as p53-phosphorylation at early time post-IR. As indicated in Fig. 6 phosphorylation of ATM- and p53 was not affected at 15 min post-IR but phosphorylation of Smad2 was completely inhibited at this time point. Likewise, this early but TGFβ-1 dependent phosphorylation of ATM and its downstream targets (i.e., p53, chk2) is further supported by the use of the TGFβ-1 antisense A549 subclones (see Fig. 3). By blocking transcription of TGFβ-1 a similar decrease in ATM, p53 and chk2 phosphorylation was observed directly after and up to 60 min post-IR. Thus, especially with respect to our data obtained after TβRI inhibition our results of a TGFβ-1-dependent fast stimulation of ATM and p53 phosphorylation in irradiated cells are most likely not the consequence of the Smad-signalling pathway activated by extracellular TGFβ-1 as proposed by Kirshner et al. [22].

Combining the data sets provided by Kirshner et al. [22] and our results it can be postulated that the TGFβ-1 regulated activation of DNA damage response and cell cycle control of irradiated cells involve a slow TβRI-dependent and a fast TβRI-independent mechanisms: the exact pathway of TGFβ-1 dependent but TβRI independent stimulation of ATM and p53 activity involved is currently subject of ongoing experiments.

Conflict of interest statement

The authors do not have any financial or personal relationship with other people or organisations that inappropriately influence their work.

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