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Original Research Article

In cancer cell lines inhibition of SCF/c-Kit pathway leads to radiosensitization only when SCF is strongly over-expressed

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ABSTRACT

Background and purpose: The SCF/c-Kit pathway is often overexpressed in human tumors leading to an enhanced tumorigenesis, proliferation and migration. It was now tested for NSCLC and prostate cancer cells growing in 2D and 3D whether the inhibition of this pathway can be used to achieve a significant radiosensitization and whether a respective biomarker may be identified.
Material and methods: Experiments were performed with different cancer cell lines (NSCLC: H23, H520, H226, H1975 and PrCa: DU145) growing either under 2D or 3D conditions. Expression of SCF and c-Kit was determined by RT-PCR and Western blot, SCF was knocked down by siRNA, c-Kit was inhibited by ISCK03 inhibitor and cell survival was determined by colony formation assay.
Results: There is a profound variation in the expression of both c-Kit and SCF with no association between each other. Neither levels did correlate with the respective cellular radiosensitivity determined for 2D or 3D with only a trend seen for SCF. Knock-down of SCF was generally found to result in no or only minor reduction of plating efficiency or cellular radioresistance. A significant reduction was only obtained for H520 cells characterized by an extreme over-expression of SCF. The inhibition of c-Kit by a specific inhibitor was also found to result only in minor radiosensitization.
Conclusion: Generally, the SCF/c-Kit pathway does not have a dominant effect on both, cell survival and radioresponse and, as a consequence, knockdown of this pathway does not result in a strong effect on radioresistance, except when SCF is strongly over-expressed.

Introduction

The tyrosine kinase receptor c-Kit and its ligand stem cell factor (SCF) are important key players promoting fundamental cellular functions as cell growth, survival, and migration [1]. Especially during embryonal development, the SCF/cKit axis mediates important signals for the hematopoiesis, central nervous system, intestine melanogenesis [2]. In adult tissues c-Kit is normally down-regulated, except in hematopoietic stem/progenitor cells of the bone marrow, melanocytes, and mast cells [3,4].

An active SCF/c-Kit pathway is also often seen in tumors and in pre-cancerous lesions identifying c-Kit as a proto-oncogene [1,5–12]. This activity was shown to have an oncogenic potential by driving tumor cell proliferation, migration and cancer stemness [7–12]. In line with this, for many tumors high level of SCF/c-Kit were found to be associated with poor prognosis [11–15].

Due to these findings targeting of SCF/c-Kit pathway was considered to be an optimal tool for a tumor specific treatment. Interest in this strategy was further enhanced, after detecting that SCF/c-Kit pathway is also blocked by imatinib – formerly known as an optimal inhibitor of the c-Abl tyrosine kinase [16]. Initially imatinib was used to treat leukemia [17,18] and GIST-tumors [19,20] but in recent years also for other solid tumors. There are now numerous reports showing that growth of solid tumors and metastasis are suppressed when SCF/c-Kit pathway is blocked by imatinib [21–24].

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However, there are increasing data indicating that it is unlikely that SCF/c-Kit inhibitors alone can lead to tumor cure, because of the resistance mechanisms initiated during treatment [25]. As a consequence, treatment with SCF/c-Kit inhibitors need to be combined with other efficient tools such as radiotherapy to achieve better outcomes. This combination may even have an additional benefit, when – besides the additive effect of both treatments – targeting of SCF/c-Kit will interfere with radioresponse and thereby resulting in an enhanced cellular radiosensitivity.

It is already known that the activation of c-Kit by SCF may lead to a radioresistance [26–28] considering this as an optimal tool for radioprotection, when whole body irradiation is required. On the other hand, data available so far, when SCF/c-Kit pathway is blocked, are conflicting. In a first report Holdhoff et al. [29] found that inhibition of cKit by imatinib leads to an enhanced radiosensitivity of human glioblastoma cells RuSi-RS1 but not for the human breast cancer cell line BT20 or colon cancer cells WiDr. In other reports with human glioblastoma [30] or astrocytoma cell lines [31] only a modest radiosensitization was seen after treatment by imatinib. In contrast, for RT112 bladder carcinoma cells a robust radiosensitization was obtained when treated by imatinib [32]. Overall, these data demonstrate that the radiosensitization achieved by the inhibition of SCF/c-Kit strongly varies with the cell line treated. Obviously there is a strong need to identify a biomarker allowing to identify those cell line and with that the respective tumors which will respond to this treatment, before this strategy may be applicable in clinical routine.

The study presented here was performed with ten different tumor cell lines derived either from NSCLC or prostate cancer. It was previously already shown by us, that targeting of the SCF/c-Kit pathway is an optimal tool to inhibit the metastatic potential as tested for both 2D and 3D conditions [33]. Therefore we now tested whether the same strategy can also be used to reduce radioresistance. The cell lines used were extensively characterized in respect to the SCF/c-Kit-pathway and for further analysis, five of these cell lines were selected covering the broad range of this pathway activity generally seen in tumors. Downregulation of SCF/c-Kit pathway prior to irradiation was performed either by siRNA targeting of SCF or by the specific inhibitor *ISCK03*. Again the experiments were performed for cells grown in 2D and 3D to consider the impact of growth condition on these treatments.

Material and methods

Cell culture

Experiments were performed with the human NSCLC adenocarcinoma cell lines H23 (CRL-5800), H1975 (CRL-5908), H460 (HTB-177), H1299 (CRL-5803), A549 (CCL-185), the human NSCLC squamous cell lines H226 (CRL-5826), H520 (HTB-182), the human prostate cancer cell lines DU145 (HTB-81), PC3 (CRL-1435), LnCAP (CRL-1740) obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HUVECs were isolated and used as described previously [8]. Cells were maintained in RPMI-1640 (E15-840; PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS, Greiner BioOne, Frickenhausen, Germany) at 37 °C in humidified atmosphere containing 5% CO_2. HUVECS were maintained in endothelial cell growth medium containing 5% fetal calf serum (PromoCell GmbH; Heidelberg, Germany).

For three-dimensional (3D) conditions cells were grown in 0.5 mg/ml laminin-rich extracellular matrix (lr-ECM; BD Matrigel Matrix, BD Bioscience, Bedford, MA) in agarose coated wells as described previously [33].

X-irradiation

For X-irradiation, a 6-MeV X-ray beam generated by a clinical linear accelerator was used. Irradiation was delivered at room temperature with dose rate of 4 Gy/min. Cell culture flasks were arranged between 15 mm water-equivalent plates to generate doses maximum in the cell layer.

Colony formation assay under 2D and 3D cell culture conditions

For colony formation under 2D-conditions cells were seeded in 6 cm culture dishes 24 h prior to photon-irradiation (0–8 Gy), incubated for 10–14 days, fixed, stained (0.1% crystal violet) and colonies >50 cells were counted. For 3D, cells were grown in 0.5 mg/ml lr-ECM for 24 h prior to photon-irradiation. Cell clusters >50 cells were microscopically counted 10–14 days after seeding. Plating efficiencies (PE) and survival fractions (SF) were calculated as published previously [8,34].

RNA interference by synthetic siRNA

For knockdown of SCF commercially available siRNA was used (Biomers.net GmbH, Ulm, Germany). Transfection was performed with Lipofectamine™ 2000 (final concentration 100 nM; Invitrogen-Life Technologies GmbH, Darmstadt, Germany). The forward sequences were as follows: si-SCF, UGAAGAGGAUAAUGAGAUA, si-control, UAGCGACUAACAUCAA.

Treatment with the SCF/c-Kit inhibitor ISCK03 (CAS 945526-43-2)

The Stem-Cell Factor/c-Kit Inhibitor ISCK03/CAS 945526-43-2ISCK03 inhibitor (569615, Merck Millipore, Darmstadt, Germany) was prepared and diluted 1:1000 with DMSO and again 1:1000 with medium before usage according to manufacturer instructions. Cells were incubated with 1 µl/ml ISCK03 18 h prior and again just before irradiation; control group was treated with DMSO only. For 3D the inhibitor was added to the cell and lr-ECM suspension in the same concentration.

RNA extraction and quantitative RT-PCR

Under 2D conditions RNA extraction with TriFast (peqGOLD TriFast; PeqLab Biotechnology GmbH, Erlangen, Germany) was performed according to manufacturer instructions. Under 3D conditions cells from ECM were transferred to a cup, washed with PBS (2 times) and resuspended with TriFast. RT-PCRs were performed as described previously [24]. Primer sets (F, forward; R, reverse) were: SCF, F: 5′-GGA TGG ATG TTT TGC CAA GT-3′, R: 5′-TCT TTC ACG CAC TCC ACA AG-3′; C-KIT, F: 5′- CCG GTC GAT TCT AAG TTC TAC-3′, R: 5′-GAT TGG TGC TCT CTG AAA TCTG-3′; PBGD, F: 5′-CAG CTT GCT CGC ATA CAG AC-3′, R: 5′-GAA TCT TGT CCC CTG TGG TG-3′.

Western blot analysis

Cells were rinsed with ice-cold PBS prior to adding modified RIPA buffer for protein isolation. After SDS–PAGE and transfer of proteins onto a polyvinyl difluoride (PVDF) membrane; nonspecific sites were saturated with 5% milk. Incubation was performed overnight (4 °C) with the following primary antibodies: anti-c-KIT purified rabbit anti-human (3074), anti-phospho-c-Kit (Tyr719) purified rabbit anti-human (3391), dilution 1:1000 (Cell Signaling Technologies, Danvers, USA). Immunodetection was performed by incubation (1 h) with peroxidase-conjugated secondary antibodies: goat anti-rabbit IgG (H + L) cross adsorbed secondary antibody (31462), dilution 1:2000 (Pierce-ThermoFisher Scientific,

Rockford, IL, USA), with a self-made ECL system. Western blot signals were quantified by densitometric scanning (Bio Rad ChemiDoc XRS+, Bio-Rad Laboratories, Inc., Hercules, USA).

ELISA for SCF

SCF protein levels were measured in 2D cell culture supernatants with a commercial ELISA kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instruction. Biotinylated secondary antibody and streptavidin conjugated horseradish peroxidase were used for detection of captured SCF by measuring absorbance at 450 nm, using a 96-well plate spectrophotometer (BioTek, VT, USA). SCF protein is expressed in pg/ml.

Calculation and statistical analyses

Data shown are mean values ±standard errors of the mean (SEM) for at least 3 independent experiments. The level of significance was evaluated by Student's *t*-test. Differences at p values of <0.05 were considered statistically significant and are indicated in the figures by an asterisk.

Results

Huge differences of c-Kit and SCF in NSCLC and prostate cancer cell lines

Initially, we assessed the status of the c-Kit pathway of eight different human non small cell lung cancer cell lines (H23, H226, H1975, H460, H1299, H520, A549) and three human prostate cancer cell lines (DU145, PC3, LnCAP) by measuring the expression of the stem cell factor (SCF), which is the natural ligand of the c-Kit pathway (Fig. 1A), and of c-Kit (Fig. 1B) for both 2D and 3D cultures using RT-PCR. Expression is plotted as ΔΔCt-values relative to the expression of the human umbilical vein endothelial cell line HUVEC. For both genes, marked variations are seen with, however, no obvious differences between 2D and 3D cultures. For SCF an extreme over-expression was only seen for H520 cells, which was by a factor of 25–32 higher than measured for the other cell lines. For four cell lines we also measured the SCF protein expression using ELISA (Supplement Fig. S1). Although, there was a clear correlation between both parameters, it was noted that the scatter was much larger for the protein level, especially when the level was low. Therefore, in case of SCF the measurement of mRNA was considered to be more robust when compared to the protein expression as determined by ELISA.

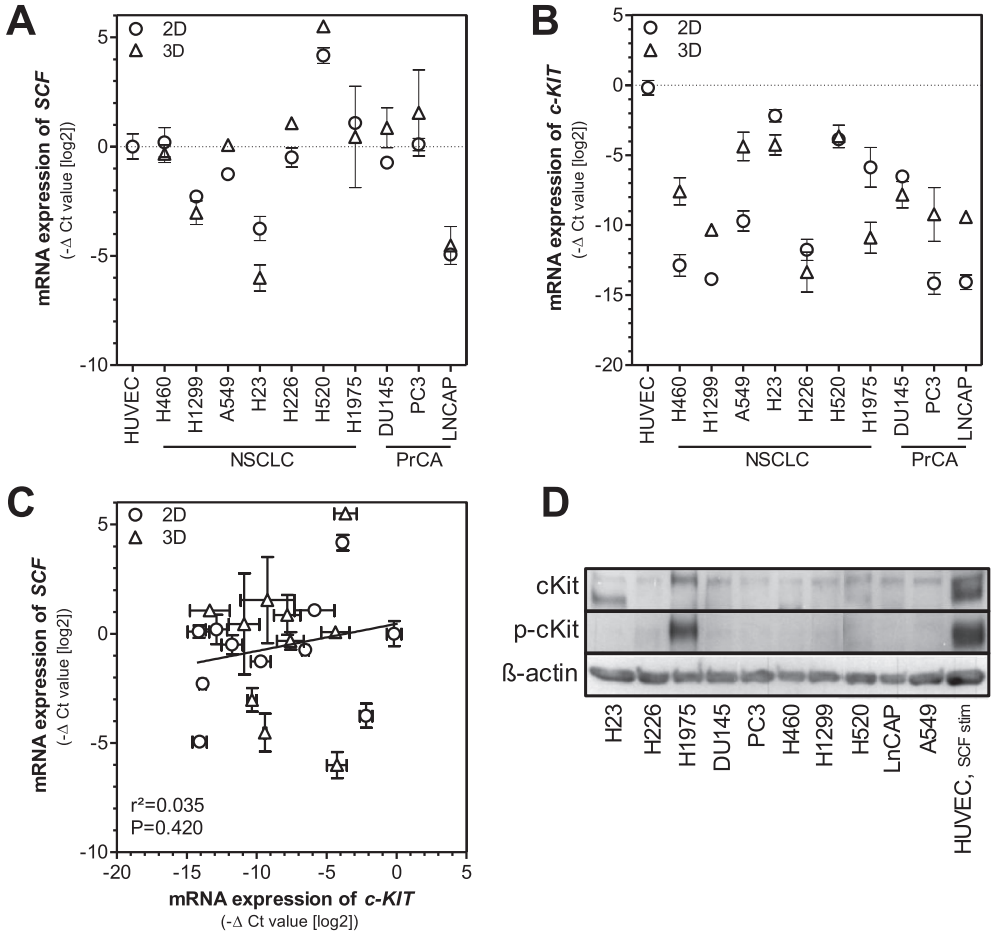

Fig. 1. Characterization of SCF/c-Kit pathway in NSCLC (H460, H1299, A549, H23, H226, H520, H1975) and prostate cancer cell lines (DU145, PC3, LnCAP) grown either under 2D or 3D conditions. (A and B) Variation of mRNA expression for either SCF or c-Kit as determined by RT-PCR, which is expressed as −ΔCT values using HUVEC cells grown in 2D as a reference. (C) Association between the expression of SCF and c-Kit. D) Variation of c-Kit and its phosphorylated isoform phospho-c-Kit (p-c-Kit) as determined by western blotting with ß-actin used as loading control. Values are means + SEM, $n \geq 3$.

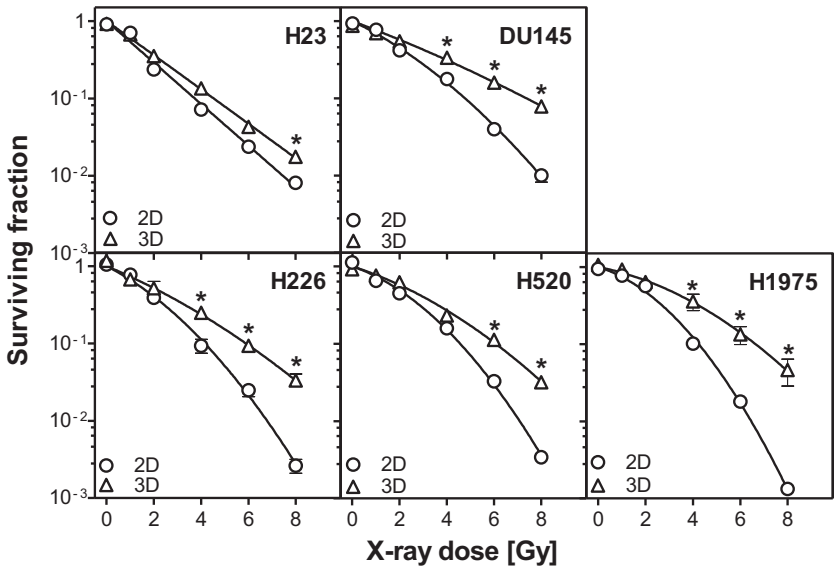

Fig. 2. Variation in the cellular radiosensitivity of NSCLC and prostate cancer cell lines grown either in 2D or 3D conditions. Cells were irradiated with increasing photon doses from 0 to 8 Gy without any further treatment. Values are means ± SEM, $n \geq 3$; significant differences ($P < 0.05$) are indicated by asterisks; other differences were not significant different.

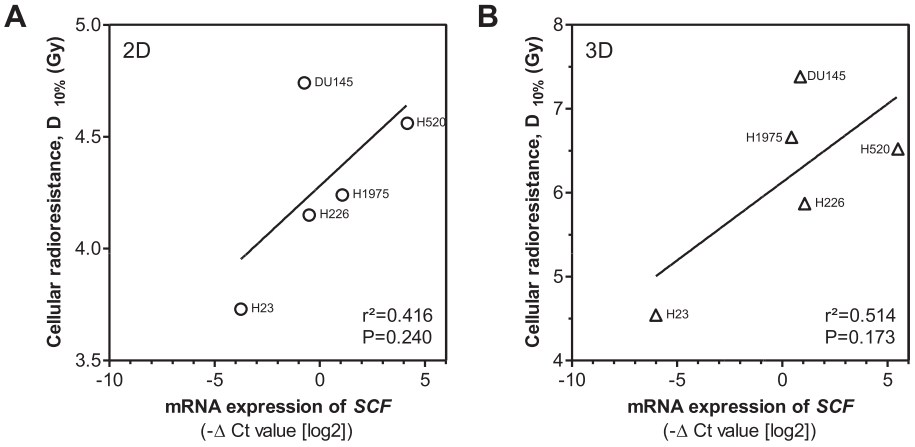

Fig. 3. Association between the cellular radioresistance and the expression of SCF for NSCLC and prostate cancer cell lines grown either in 2D or 3D conditions. Cellular radioresistance is expressed as X-ray dose, $D_{10\%}$, required to reduce survival to 10% as determined from data presented in Fig. 2; expression of SCF was taken from Fig. 1A. (A) 2D; (B) 3D condition. Data were analysed by linear regression analysis.

Overall, the data shown in Fig. 1 indicate that the expression of both SCF and c-Kit strongly varies with the cell line tested but does not depend on growth condition. For these two levels there was also no association between each other (Fig. 1C) indicating that the activity of the SCF/c-Kit pathway is regulated separately by both, SCF and c-Kit.

For c-Kit we determined the protein expression as well as its phosphorylated isoform phospho-c-Kit (p-c-Kit) for cells grown in 2D and without SCF stimulation (Fig. 1D). The status of c-Kit positive cell line HUVEC stimulated by SCF was used as reference. Again, there are marked differences in both levels with most cell lines showing a low level of c-Kit and with a strong auto-phosphorylation only seen for H1975. In general, these are similar to those detected for gene expression (Fig. 1A and B). But there are also some clear outliers as seen for H23 and H520 both showing a high RNA expression for c-Kit but a low protein level. These data indicate that the level of c-Kit is not solely determined by transcription but also by post-translational processes. For further analysis five cell lines were selected (H23, H226, H520, H1975 and DU145) to cover the broad range of activity seen for the c-Kit pathway in human tumor cells.

No strong association between c-Kit pathway and cellular radiosensitivity

Fig. 2A shows the range of cellular radiosensitivity as determined for the five tumor cell lines when irradiated either in 2D or 3D. For all tumor cell lines 3D growth results in a clear increase in radioresistance as previously observed by others [35,36]. It was tested, whether this variation in cellular radiosensitivity may in part be determined by the different expression measured for SCF or c-Kit. However, for both genes no significant association was seen with only a moderate trend for SCF (Fig. 3, Supplement Fig. S1). These data indicate that the c-Kit pathway does not strongly regulate the cellular radiosensitivity of human tumor cells for both 2D as well as 3D with probably a minor impact of SCF.

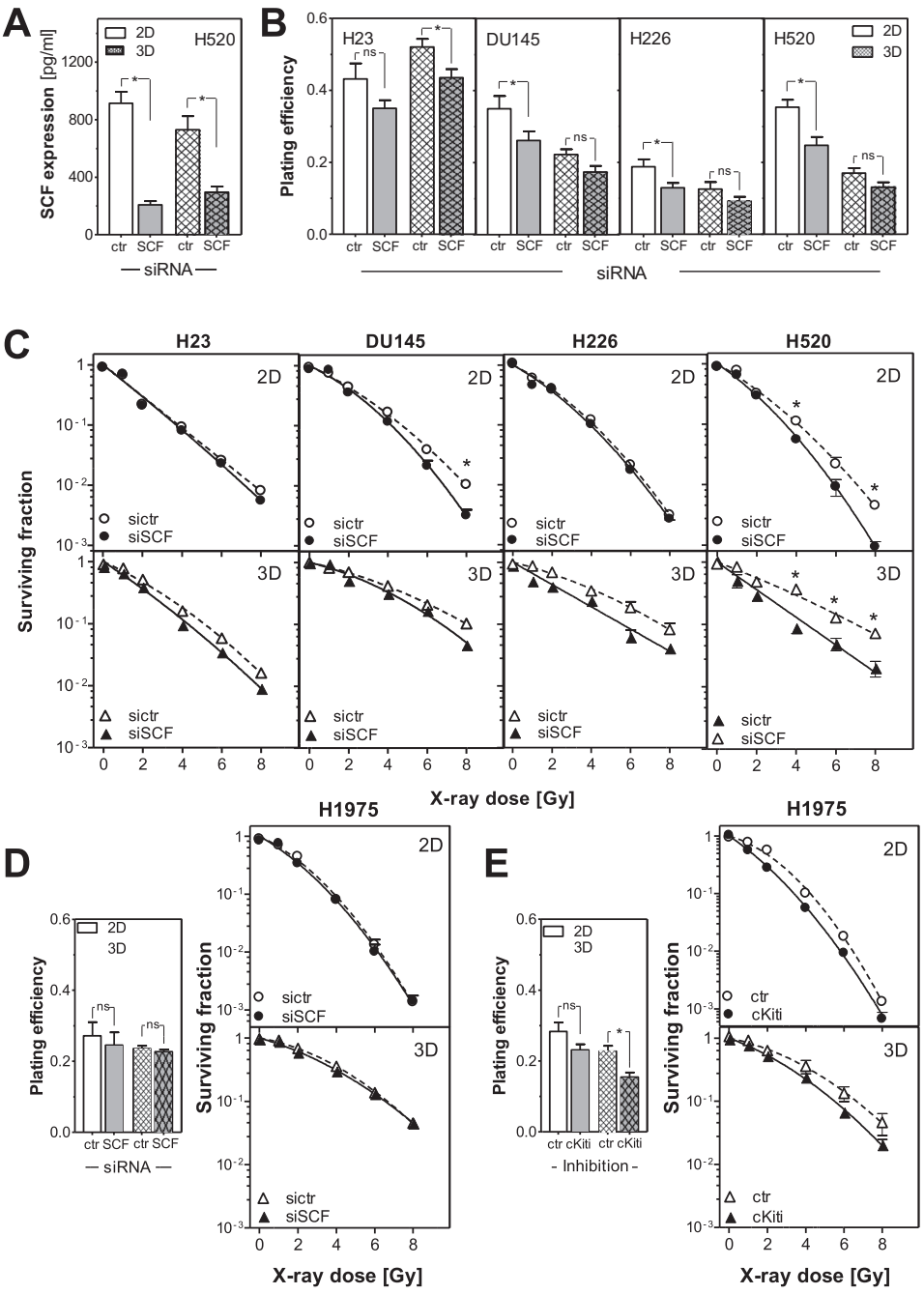

Fig. 4. Impact of SCF/c-Kit knock-down. (A) Efficiency of SCF knock down as achieved by siRNA on protein level in H520 cells grown either in 2D or 3D conditions. (B) Impact of SCF knock down by siRNA on PE and (C) on cellular radiosensitivity for cells grown either under 2D or 3D conditions. (D) Impact of SCF knockdown on PE and radiosensitivity of c-Kit auto-phosphorylated cell line H1975. (E) Impact of c-Kit inhibitor ISCK03 (CAS 945526-43-2) on PE and cellular radiosensitivity of H1975 cells. Values are means + SEM, $n \geq 3$; significant differences ($P < 0.05$) are indicated by asterisks; other differences were not significant different, ns.

Knock-down of SCF only moderately affects PE and cellular radiosensitivity

Next, we tested the effect of SCF knock-down as achieved by siRNA allowing a strong down-regulation on protein level for both 2D as well as 3D cultures (Fig. 4A). For the plating efficiency, PE, no or only marginal reduction was seen (Fig. 4B). Also for the cellular radiosensitivity only moderate effects are observed with no or only minor decrease in cell survival when SCF was knocked down (Fig. 4C). These effects appear to be slightly stronger, when cells were grown under 3D conditions. Solely for H520 cells a stringent significant radiosensitization was obtained, when SCF was knocked down for both 2D and 3D conditions. This is considered to result from the extreme over-expression of SCF as only found for this cell line (Supplement Fig. S2A). No such trend was seen when enhancement factor (EF) determined at a survival fraction of 10% was plotted vs. the protein or mRNA expression of c-Kit (Fig. S2B and C).

As expected no effect of SCF knock-down on PE or radiosensitivity was obtained for H1975 cells (Fig. 4D), since this cell line is characterized by a pronounced c-Kit auto-phosphorylation. For this cell line we also tested the effect of the specific inhibitor ISCK03 (CAS 945526-43-2), which blocks the c-Kit (auto)-phosphorylation [37]. Again, only a moderate effect on both PE and radiosensitivity was obtained (Fig. 4D).

Overall these data indicate that generally in NSCLC and PrCa cell lines proliferation and cellular radiosensitivity are not strongly determined by the SCF/c-Kit pathway and, as a consequence, blockage of either SCF or c-Kit has no or only a minor effect on these parameters, except for an extreme over-expression of SCF as found for H520 cells.

Discussion

So far data about radiosensitization when targeting SCF/c-Kit pathway are diverse ranging from no up to very strong effects with, so far, no parameter available to predict this response.

In our study 10 different tumor cell lines derived from NSCLC or prostate cancer were used in order to identify a biomarker allowing such a prediction. For these 10 cell lines a detailed characterization of the SCF/c-Kit pathway was performed revealing that there is a broad variation in the mRNA expression of SCF and c-Kit as well as the protein level of c-Kit and p-c-Kit (Fig. 1). Similar observations were previously made by others for pancreatic [11], NSCLC [38] or prostate cancer cells [39]. Beside this variation we observed that there were always one or two cell lines with extreme values, as for instance for H520 cells characterized by an extreme over-expression of SCF, and H23 and LnCap both showing a very low expression when compared to the other cell lines (Fig. 1A). And also for c-Kit a substantial variation was measured with H23 and H1975 cells having high levels of c-Kit and H1975 even a strong auto-phosphorylation (Fig. 1B and D). We also describe that there is no association between these two mRNA expression levels (Fig. 1C) as previously also observed for tumor samples taken from adenoid cystic carcinoma [45]. This is in line with recent histological data obtained for 72 NSCLC tumor biopsies showing that immunopositivity for both KIT and SCF (KITLG) in the same tumor was rare [46]. Overall these data indicate that in human tumors there appears to be no stringent pattern indicating an aberrant SCF/c-Kit activity but rather a very heterogeneous picture as previously suggested by others [40].

The five cell lines selected for further analysis were found to show a broad range in radiosensitivity for both 2D and 3D conditions (Fig. 2) with cells grown in 3D being more resistant as previously reported by others [29]. This radiosensitivity was obviously not correlated with the respective expression of c-Kit (Fig. 1S). For SCF there appears to be a trend with cell lines being more radioresistant when expressing high levels of SCF. This result is in line with previous data showing that cellular radioresistance is generally enhanced when adding SCF [26,28,41].

The effect of SCF knock-down by siRNA on radiosensitivity was measured to be quite diverse ranging from no up to a significant sensitization (Fig. 4C). This is in line with data observed by others [26–32]. A significant sensitization was only found for H520 cells (Fig. 4C). This cell line is characterized by a strong over-expression of SCF (Fig. 1C) suggesting again a possible correlation between SCF and radioresistance as already mentioned above. In line with this, after SCF knockdown no distinct radiosensitization was seen for cell lines with low or only moderate level of SCF (Figs. 3C and S2). Overall these data suggest that a strong over-expression of SCF may be a potential biomarker for a significant radiosensitization achievable when targeting the SCF/c-Kit pathway. This criteria, however, was only met in one out of the ten tumor cell lines tested. An even lower frequency was seen in a study with adenoid cystic carcinoma, where SCF expression was also determined with RT-PCR, with only 1 out of 27 samples showing a strong over-expression of SCF [45]. In other studies with tumor samples expression of SCF was generally determined via immunohistochemistry, which only allows a semi-quantitative estimation but not to identify tumors with an extreme over-expression [46,13,38]. Clearly more data is needed on this subject.

Previously Oertel et al. [30] suggested that the extent of radiosensitization obtained when targeting SCF/c-Kit pathway by imatinib depends on the protein level of c-Kit. However, in our study no obvious association between level of c-Kit and increase in radiosensitivity was seen when targeting SCF/c-Kit pathway (Fig. 2SB and C). The identical observation was made by Holdhoff et al. [29] and Moura et al. both [42] using imatinib to block the SCF/c-Kit pathway. The strong need for such a biomarker was recently also highlighted by the first prospective study testing the combination of radiotherapy and imatinib due to the very infrequent response reported [20].

The mechanisms by which targeting of SCF/c-Kit pathway may lead to enhanced radioresponse are still unclear. Previous data suggest that reduced phosphorylation of PDGFR [43] as well as of Akt [21,44] is of relevance but also the down-regulation of the homologous recombination protein RAD51 [32].

For tumor therapy targeting of SCF/c-Kit was also considered to be of great relevance due to its negative effect on metastasis [21–24]. However, also for this endpoint a robust biomarker is not known so far.

Overall, the relevance of SCF/c-Kit targeting either by imatinib or other inhibitors for radiotherapy would be strongly enhanced when knowing the biomarkers for both radiosensitization and prevention of metastasis. Data shown here indicate that a strong over-expression of SCF may be a biomarker for a potential radiosensitization. It should, however, be kept in mind, that the frequency of tumors matching to these criteria appears to be rather low as indicated above. Data presented here does also not recommend targeting of SCF/c-Kit by the specific inhibitor ISCK03 as a reasonable alternative, because even for a cell line with a strong auto-phosphorylation only a fairly moderate radiosensitization was obtained (Fig. 4D).

Conclusion

Our study indicates that generally targeting of the SCF/c-Kit pathway will only have a minor effect on radiosensitivity, except when SCF is strongly over-expressed.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ctro.2017.02.001.

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