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Original article

Cancer cell motility is affected through 3D cell culturing and SCF/c-Kit pathway but not by X-irradiation

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ABSTRACT

Background and purpose: Success of radiotherapy is often limited by therapy resistance and metastasis resulting from cancer cell motility. It was tested *in vitro* whether this cancer cell motility is affected by growth condition, active SCF/c-Kit pathway or X-irradiation.

Materials and methods: Cell motility was measured with BioCoat™ Matrigel™ invasion chamber using four different cancer cell lines (NSCLC: H23, H520, H226 and PrCa: DU145). Cells were grown in 2D or 3D, SCF was knocked down by siRNA and cells were irradiated with 2 or 6 Gy.

Results: All cell lines except H520 showed a 2–3-fold increase in cell motility when grown in 3D. This effect was considered to result from the EMT-like change seen when cells were grown in 3D as indicated by the enhanced expression of vimentin and N-cadherin and reduction of E-cadherin. Just the opposite trends were found for H520 cells. Knockdown of SCF was found to result in reduced cell motility for both 2D and 3D. In contrast, X-irradiation did not modulate cell motility neither under 2D nor 3D. In line with this, X-irradiation did neither induce the expression of EMT-associated genes nor SCF.

Conclusion: X-irradiation affects neither the expression of important EMT genes such as vimentin, E-cadherin and N-cadherin nor SCF/c-Kit signaling and, as a consequence, does not alter cell motility.

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Success of cancer therapy depends on both, local tumor control as well as the absence of metastasis. Radiotherapy is known to be an optimal tool to achieve local control. However, recent experimental data also suggest, that on the other hand X-irradiation may also stimulate cancer cell motility/invasion and metastasis [1–6].

So far, the underlying mechanisms of this effect are only partly understood. To initiate metastasis, cancer cells must initially disseminate from the primary tumor and invade through the surrounding basement membrane and stroma into lymphatic or blood vessels [7]. This process requires a so-called epithelial to mesenchymal transition (EMT), by which epithelial tumor cells lose their firm structure and are transformed into mesenchymal like cells allowing migration and invasion. This EMT is characterized by distinct changes in the expression of respective markers such as the down-regulation of E-cadherin on one side and the up-regulation of vimentin and N-cadherin on the other side [7,8].

The activation of EMT has been proposed as the critical step for the acquisition of invasiveness of epithelial cancer cells [8]. It was recently suggested that X-irradiation may affect metastasis via a stimulation of EMT by activating defined cellular and molecular mechanisms in the irradiated microenvironment [9]. Tumors transplanted into pre-irradiated mammary tissue were found to show reduced angiogenesis and more hypoxia as well as stronger invasiveness, and – most of all – a higher metastatic spread to lung and lymph nodes when compared to non-irradiated tissue [2]. It was shown that the SCF/c-Kit axis may be involved in this process due to the higher secretion of SCF by hypoxic tumor cells leading to a pronounced mobilization of c-Kit + myeloid cells from bone marrow that will home to the tumor to stimulate EMT.

The stem cell factor (SCF) binds to the tyrosine kinase receptor c-Kit [10]. During normal development, this activation plays a critical role in controlling cell migration, in particular, at sites of hematopoiesis, in the central nervous system, in the intestine, and in the melanogenesis [11]. c-Kit is normally down-regulated in adult tissues, except in hematopoietic stem/progenitor cells of the bone marrow, in melanocytes, and in mast cells. Accordingly, in adult

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cells the SCF/c-Kit axis is essential for the maintenance of hematopoiesis and for mast cell survival [12,13].

c-Kit is also known to act as an oncogene as documented for gastrointestinal stromal tumors (GIST), mastocytosis, and melanoma [14]. This effect was found to result from activating mutations in the extracellular or intracellular domain [10] as well as through an autocrine SCF/c-Kit loop [15].

It was now questioned here, whether – besides the c-Kit + myeloid cells mediated metastasis – X-irradiation may also affect tumor cell migration *in vitro* via an activated SCF/c-Kit signaling resulting in an enhanced EMT-like transition. Cell motility was measured *in vitro* using the 8 μm pore size BD BioCoat™ Matrigel™ Invasion Chamber (BD Bioscience, Bedford, MA). The EMT-like transition was measured via the expression of respective marker genes such as E- and N-cadherin as well as vimentin. The experiments were performed with cancer cells grown in both 2D as well as in 3D conditions to mimic a more physiological growth condition.

Materials and methods

Cell culture

Experiments were performed with the human NSCLC adenocarcinoma cell lines H23 (CRL-5800), H226 (CRL-5826) and H520 (HTB-182) and the human prostate cancer cell DU145 (HTB-81) obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). 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 in air.

For three-dimensional (3D) conditions cells were grown in 0.5 mg/ml laminin-rich extracellular matrix (ECM; BD Matrigel Matrix, BD Bioscience, Bedford, MA) in agarose coated wells as described previously [16–19].

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, UAGCGACUAAACAUCAA.

X-irradiation

For X-irradiation, a 6-MeV X-ray beam generated by a clinical linear accelerator was used. The maximum dose rate was 4 Gy/min. X-irradiation was delivered at room temperature and applied doses ranged from 0 to 6 Gy. Cell culture flasks were arranged between 15 mm water-equivalent plates to generate doses maximum in the cell layer. Under 3D conditions cells were grown in ECM for 24 h prior to X-irradiation.

Transmigration assay for two- (2D) and three-dimensional (3D) conditions

Cell motility was determined using 8-μm pore size BD BioCoat™ Matrigel™ Invasion Chamber (BD Bioscience, Bedford, MA) according to the manufacturer's instructions. Cells kept in low serum medium (RPMI 1640; E15-840; PAA Laboratories GmbH, Pasching, Austria) containing 1% FBS (A15-101; PAA Laboratories GmbH, Pasching, Austria) were seeded on matrigel coated membranes or uncoated membranes (control) in the upper insert of the system. In 3D-assay cells were added to the upper inserts in a cell-matrigel-solution with a BD Matrigel Matrix (BD Bioscience, Bedford, MA) concentration of 0.5 mg/ml. In both, 2D- and 3D-assays the lower wells were filled with full serum medium containing 10% FBS. After incubation at 37 °C for 24 h the noninvading cells in the upper chamber were removed using moistened cotton swabs and then the invading cells on the bottom of the membrane were fixed and stained using crystal violet for 5 min at room temperature. The stained cells on the membrane were counted under light microscope (×20 magnification, Leica Microsysteme, DMIL LED, Wetzlar, Germany). For calculation of the cell motility in % the mean of cells invaded through the matrigel coated insert membrane were divided by the mean of cells migrated through the uncoated control insert membrane.

RNA extraction and quantitative RT-PCR

Under 2D conditions RNA extraction with TriFast (peqGOLD TriFast; PeqLab Biotechnology GmbH, Erlangen, Germany) was performed according to the manufacturer's 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 [20,21]. Primer sets (F, forward; R, reverse) were: Vimentin, F: 5′-GCA AAG CAG GAG TCC ACT GAG TAC C-3′, R: 5′-TGT CAA GGG CCA TCT TAA CAT TGA G-3′; N-Cadherin, F: 5′-CAA TCC TCC AGA GTT TAC TGC CAT G-3′, R: 5′-GAT TGG TTT GAC CAC GGT GAC TAA C-3′; E-Cadherin, F: 5′-TGA AAA GAG AGT GGA AGT GTC CGA G-3′, R: 5′-GAT TAG GGC TGT GTA CGT GCT GTT C-3′; SCF, F: 5′-GGA TGG ATG TTT TGC CAA GT-3′, R: 5′-TCT TTC ACG CAC TCC ACA AG-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

Under 2D conditions, cells were rinsed with ice-cold PBS prior to adding modified RIPA buffer for protein isolation. Under 3D conditions cell culture flasks were put on ice and the cells from ECM were transferred to a cup to reduce the amount of matrigel in sample and lysed using modified RIPA buffer (50 mM Tris–HCl (pH 7.4), 1% Nonidet-P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, Complete protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM NaVO4, 2 mM NaF). Protein concentration was determined with the BCA assay (Pierce-Thermo Fisher Scientific, Rockford, IL, USA).

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-Vimentin purified mouse anti-human, dilution 1:1000 (C-9080, Sigma–Aldrich, St. Louis, MO, USA), anti-β-Actin purified rabbit anti-human, dilution 1:2000 (Ab 8227, Abcam, Cambridge, UK). Immunodetection was performed by incubation (1 h) with peroxidase-conjugated secondary antibodies: goat anti-mouse IgG (H + L) cross adsorbed secondary antibody (31432) and goat anti-rabbit IgG (H + L) cross adsorbed secondary antibody 31462, dilution 1:2000 (Pierce-Thermo Fisher Scientific, Rockford, IL, USA), with a selfmade ECL system. Western blot signals were quantified by densitometric scanning (Bio Rad ChemiDoc XRS+, Bio-Rad Laboratories, Inc., Hercules, USA).

Calculation and statistical analyses

Data shown are mean values ± standard errors of the mean (SEM). The level of significance was evaluated by Student's *t*-test. Differences at p values of <0.05 were considered statistically significant.

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