

# Comparison of the proliferative and clonogenic growth capacity of wound fluid from breast cancer patients treated with and without intraoperative radiotherapy

Marlon R. Veldwijk<sup>1</sup>, Christian Neumaier<sup>1</sup>, Axel Gerhardt<sup>2</sup>, Frank A. Giordano<sup>1</sup>, Marc Sütterlin<sup>2</sup>, Carsten Herskind<sup>1\*</sup>, Frederik Wenz<sup>1\*</sup>

<sup>1</sup>Department of Radiation Oncology, <sup>2</sup>Department of Gynecology and Obstetrics, Universitätsmedizin Mannheim, Medical Faculty Mannheim, Heidelberg University, Theodor-Kutzer-Ufer 1-3, D-68135 Mannheim, Germany

\*These authors shared senior authorship to this work.

Correspondence to: Marlon R. Veldwijk, Ph.D. Department of Radiation Oncology, Universitätsmedizin Mannheim, Medical Faculty Mannheim, Heidelberg University, Theodor-Kutzer-Ufer 1-3, Mannheim D-68135, Germany. Email: Marlon.Veldwijk@medma.uni-heidelberg.de.

**Abstract:** The physiological wound healing process after breast-conserving surgery is believed to contribute to a microenvironment promoting motility and invasive activity of residual malignant cells. In intraoperative radiotherapy (IORT), a high single dose of radiation is applied to the tumor bed directly after surgical removal of the tumor and this has been reported to abrogate the stimulatory effects of wound fluid (WF). In the present study, we tested whether IORT alters the influence of WF on the proliferative and clonogenic growth of human MCF7 breast cancer cells. Breast cancer patients were recruited from our IORT studies. WF from 12 patients who underwent IORT, and 18 control patients without IORT, were collected for 24 h. Proliferation was tested in a short-term (MTT) assay and the colony formation assay. A non-significant trend for reduced proliferation was seen in the MTT assay when WF from IORT-treated patients was added at 1% ( $P=0.07$ ) but not at 3% ( $P=0.16$ ). No significant effect of IORT-treated WF on the clonogenic growth capacity of MCF7 cells ( $P=0.79$ ) was found. Our short-term proliferation results with the ER/PgR<sup>+</sup>-Her2/neu<sup>-</sup> cell line MCF7 complement previously published data with ER/PgR<sup>-</sup>-Her2/neu<sup>-</sup> and ER/PgR<sup>-</sup>-Her2/neu<sup>+</sup> breast cancer cell lines showing no significant effect of IORT on short-term proliferation. In addition, for the first time, a lack of an effect of IORT on the clonogenic growth capacity of WF was shown. It should be noted that the present results do not exclude potential other effects of IORT on the cytokine composition and functional activity of WF.

**Keywords:** Intraoperative radiotherapy (IORT); surgery; wound fluid (WF); cell proliferation; clonogenic cell assay

Submitted Jan 23, 2015. Accepted for publication Mar 31, 2015.

doi: 10.3978/j.issn.2218-676X.2015.04.01

View this article at: <http://dx.doi.org/10.3978/j.issn.2218-676X.2015.04.01>

## Introduction

The ability of the cellular microenvironment to influence cell behavior has been known for quite some time. In malignancies, the microenvironment was shown to regulate tumor cell fate even suggesting that disruption of its homeostasis may drive tumor progression (1). Induction of the wound healing response after surgery with the ensuing microenvironment reorganization and tissue reconstruction may, therefore, potentially influence

recurrence (2). After damage, wound healing, including inflammation, tissue repair, and remodeling, is essential to ensure host integrity in multicellular eukaryotic organisms (3). As previously shown in experimental systems (4,5), it is to be expected that growth factors secreted during wound healing can also affect growth of malignant and non-malignant cells clinically. It was previously observed that wound fluid (drained after surgery; WF) collected from breast cancer patients can indeed stimulate proliferation of breast cancer cells (6).

In intraoperative radiotherapy (IORT), a high single dose of radiation is applied to the tumor bed directly after surgical removal of the tumor, in contrast to conventional external-beam radiotherapy (EBRT) which is applied after wound healing is completed. In a previous study, WF obtained from patients treated with IORT within the TARGIT-A trial (7) was reported to produce a reduction in WF-stimulated proliferation and invasion of breast cancer cell lines *in vitro* compared to WF from non-IORT patients (8,9). However, no significant effect of IORT on the proliferative capacity of WF was observed in a short-term proliferation assay (2-D) using ER/PgR<sup>-</sup>-Her2/neu<sup>-</sup> and ER/PgR<sup>-</sup>-Her2/neu<sup>+</sup> breast cancer cell lines. Furthermore, although significant effects of IORT were found in invasion (3-D Matrigel) and migration assays, clonogenic proliferation was not tested.

Therefore, the purpose of the present study was to validate the effect of IORT on WF-stimulated short-term proliferation in an ER/PgR<sup>+</sup>-Her2/neu<sup>-</sup> human breast cancer cell line (MCF7), and for the first time test the effect on clonogenic, long-term proliferation.

## Methods

### Cell culture

The human breast carcinoma cell line MCF7 (ER/PgR<sup>+</sup>-Her2/neu<sup>-</sup>; American Type Culture Collection, LGC Standards GmbH, Wesel, Germany) was propagated in DMEM supplemented with 10% fetal bovine serum (FBS; all from Biochrom AG, Berlin, Germany). Three days before each experiment, cells were cultured in 3% FBS-containing medium. Cells were kept at 37 °C in a humidified incubator with 95% air/5% CO<sub>2</sub>.

### Collection and preparation of WF

Thirty patients with low-risk breast cancer were treated with breast-conserving surgery, of which 12 received IORT with a single dose of 20 Gy prescribed to the applicator surface (10-12). After surgery, WF was drained from the wound for 24 h. Thereafter, WF samples were collected, centrifuged at 800 ×g for 5 min and the supernatant was filtered through 40 μm filters (BD Falcon, Heidelberg, Germany). After a second centrifugation step (3,500 ×g for 5 min), the supernatant was subsequently filtered through 5, 0.8 and 0.22 μm filters and aliquots stored at -80 °C. These steps ensured sufficient removal of cells and debris from the WF that would otherwise interfere with cell growth. The study was approved by the Medical Ethics Commission II of

the Medical Faculty of Mannheim, Heidelberg University and was conducted according to Declaration of Helsinki principles. Of note, although all patients received a perioperative antibiotic treatment, this was prolonged (3 days) for patients receiving IORT *vs.* a single application for non-IORT-treated patients.

### Proliferation assay

MCF7 cells were seeded in 96 well plates (8 wells per group, 5×10<sup>3</sup> cells per well in 50 μL serum-free DMEM medium). Samples were supplemented with 50 μL DMEM medium containing 3% FBS and 1% or 3% WF. After 48 h, 20 μL MTT [5 mg/mL, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and incubated for 3 h at 37 °C. Viable cells reduce the yellow MTT to a non-hydrosoluble purple formazan. Thereafter, 100 μL of 10% SDS (Sodium dodecyl sulfate)/10 mM HCl in PBS was added to each well and plates incubated o/n at 37 °C to allow dissolving of the formazan. The next day, the absorbance at 590 nm (reference 690 nm) was quantified using a spectrophotometer (Tecan Infinite M200). Results are shown as percentage of controls.

### Colony formation assay

MCF7 cells were seeded at 150 cells/T25 culture flask (triplicates) in 4 mL DMEM medium supplemented with 3% FBS and 3% WF and incubated for 2 weeks in a humidified incubator with 95% air/5% CO<sub>2</sub> at 37 °C. Thereafter, cells were fixed with methanol/acetic acid and stained with crystal violet as described previously (13). Colonies (≥50 cells) were scored and the plating efficiency determined: plating efficiency = number of colonies obtained/number of cells seeded.

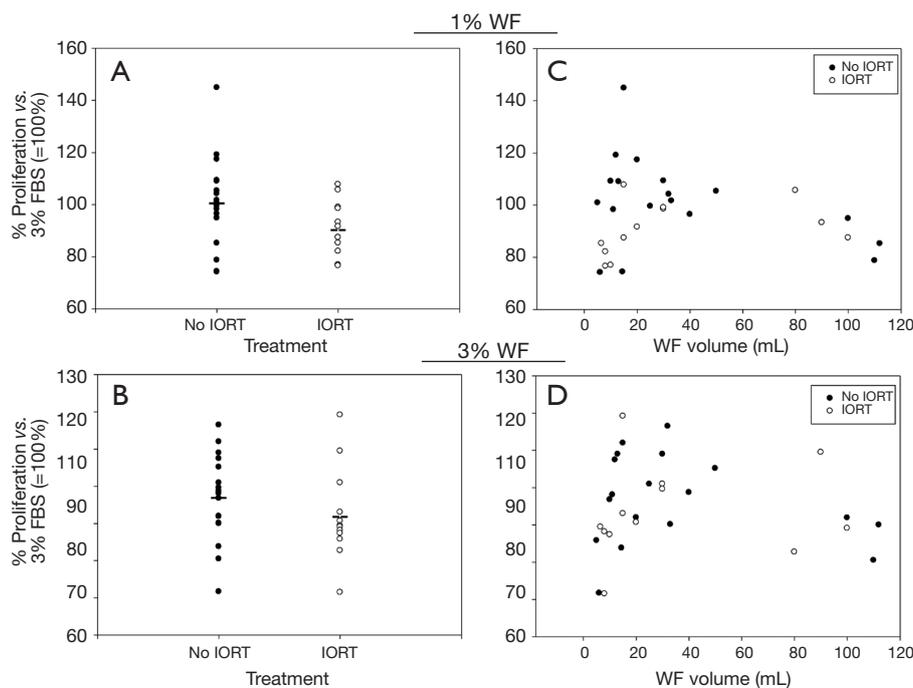
### Statistics

Replicates were performed at least in triplicate and data are presented as mean ± standard error, unless otherwise noted. Wilcoxon/Kruskal-Wallis (non-parametric) tests and linear regression were performed with JMP11 statistical software (SAS Institute GmbH, Böblingen, Germany). Graphs were plotted using SigmaPlot 11.0 (Systat Software GmbH, Erkrath, Germany).

## Results

### Effects of IORT on the short-term proliferative capacity of WF

Pilot experiments showed that MCF7 cells did not



**Figure 1** Proliferation of MCF7 cells (MTT assay) after 48h incubation with WF from breast cancer patients treated with or without IORT. Cells were either treated with 1% (A,C) or 3% WF (B,D). All samples were treated with 3% FBS which was also used as control (3% FBS=100%). Whereas for 1% WF a strong (A.  $P=0.07$ ) and for 3% WF a weak trend (B.  $P=0.16$ ) for an inhibitory effect of IORT on the proliferative capacity of WF could be observed, for neither group this was significant. In addition, it was tested whether there was a correlation between the WF volume and the effect of WF on the proliferation (C,D). Here no significant correlation between the proliferation rates and the WF volume was observed (C. 1% WF:  $R^2_{No\ IORT}=0.138$ ,  $R^2_{IORT}=0.119$ ; D. 3% WF:  $R^2_{No\ IORT}=0.045$ ,  $R^2_{IORT}=0.015$ ). Please note the scaling of the graphs.  $n_{No\ IORT}=18$ ,  $n_{IORT}=12$ . IORT, intraoperative radiotherapy; WF, wound fluid; FBS, fetal bovine serum.

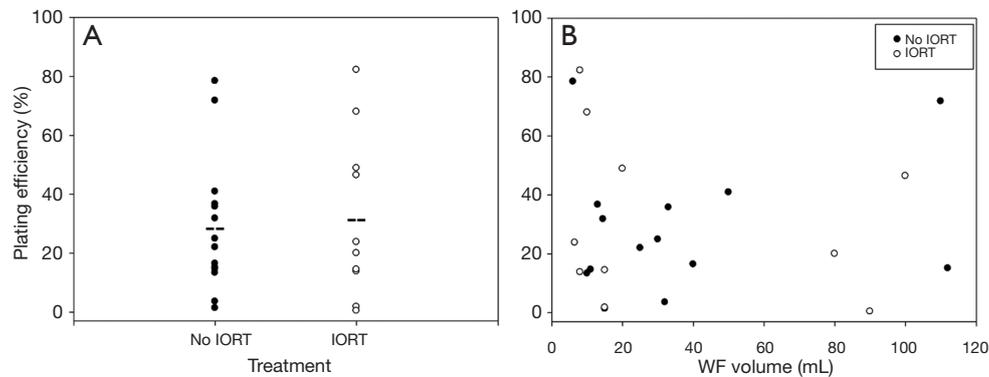
proliferate when supplemented with pure WF and that 3% FBS was required to warrant proper proliferation (data not shown). To test potential concentration effects, experiments were performed with either 1% or 3% WF added to medium containing 3% FBS and normalized to the proliferation rates of cells receiving only 3% FBS. For 1% WF a trend for a modest inhibiting effect of IORT on the proliferative capacity of WF was observed ( $P=0.07$ ; no IORT:  $101.3\% \pm 4.0\%$  vs. IORT:  $91.0\% \pm 3.0\%$ ; *Figure 1A*) and the difference was also not significant using 3% WF ( $P=0.16$ ; no IORT:  $97.4\% \pm 2.8\%$  vs. IORT:  $92.0\% \pm 3.6\%$ ; *Figure 1B*). As large variations in the volumes of the WF occurred, a potential correlation between the volume and the effect on proliferation of MCF7 cells was tested. Here, no correlation could be detected, irrespective of using 1% ( $R^2_{No\ IORT}=0.138$ ,  $R^2_{IORT}=0.119$ ; *Figure 1C*) or 3% WF ( $R^2_{No\ IORT}=0.045$ ,  $R^2_{IORT}=0.015$ ; *Figure 1D*).

### ***Effects of IORT on the clonogenic growth capacity of WF***

As repopulation of residual tumor cells to form recurrences depends on the capacity of cells to reproduce themselves, the effect of IORT on WF-stimulated clonogenic growth was tested in the colony formation assay. 3% WF from patients receiving IORT had no significant effect on the plating efficiency after 14 days incubation compared to that of WF from patients not receiving IORT ( $P=0.79$ ; no IORT:  $29.0\% \pm 6.1\%$  vs. IORT:  $32.0\% \pm 8.9\%$ ; *Figure 2A*). Also, no significant correlation between the volume of the WF and the plating efficiency was observed ( $R^2_{No\ IORT}=0.04$ ,  $R^2_{IORT}=0.05$ ; *Figure 2B*).

### **Discussion**

In this work the effect of IORT on the proliferation and clonogenic growth capacity of WF obtained from breast cancer patients treated with or without IORT was investigated. Using 1% WF, a non-significant trend for an



**Figure 2** (A) Clonogenic proliferation of MCF7 cells after 14 day incubation during colony formation with 3% WF from breast cancer patients treated with or without IORT. (B) All cells were treated with 3% WF and 3% FBS. No correlation between the WF volume and the effect of WF on the plating efficiency was observed. For none of the groups, a significant difference between the effect of IORT or lack of IORT on clonogenic growth (A.  $P=0.79$ ) or a correlation between the clonogenic growth and the WF volume was observed (B.  $R^2_{\text{No IORT}}=0.04$ ,  $R^2_{\text{IORT}}=0.05$ ).  $n_{\text{No IORT}}=14$ ,  $n_{\text{IORT}}=10$ . IORT, intraoperative radiotherapy; FBS, fetal bovine serum; WF, wound fluid.

inhibiting effect of the IORT on the proliferative capacity was observed (*Figure 1A*) but not for 3% WF ( $P=0.16$ ; *Figure 1B*). Similarly, no significant effect of the IORT on the clonogenic growth capacity of WF was observed either (*Figure 2A*). Our results from the short-term proliferation assay (MTT) with ER/PgR<sup>+</sup> and Her2/neu<sup>-</sup> MCF7 cells complement previous data by Belletti *et al.* (8) on MDA-MB-231 (ER/PgR<sup>-</sup>-Her2/neu<sup>-</sup>), MDA-MB-453 and SKBR-3 (both ER/PgR<sup>-</sup>-Her2/neu<sup>+</sup>) and are broadly in line with the absence of a significant effect of IORT on WF-stimulated proliferation in 2-D cultures in these cell lines. Although stimulation of proliferation by WF was found to be higher in Her2/neu positive than in negative cell lines (6), IORT did not seem to have a significant effect on the proliferative capacity of WF irrespective of the estrogen, progesterone, and Her2 receptor status of the breast cancer cell line. However, it should be noted that in the present study MCF7 cells did not proliferate when supplemented with pure WF but required addition of 3% FBS in both assays. This is consistent with recent evidence from head and neck tumor cell lines that the stimulatory effect of WF on proliferation may be cell-line specific (14).

As WF volumes varied greatly, we speculated that this may potentially modulate the effects of the WF on the tested proliferation and clonogenic growth of MCF7 cells. The rationale behind this was that increased/decreased content of diluting liquid (blood/ lymph) will affect the concentration of growth modulating molecules in the WF. No correlation was detected between the WF volume and proliferation of MCF7 cells in either assay (*Figure 1C,D* and *Figure 2B*), thereby arguing against this hypothesis.

A limitation of the present study may be that WF

did not stimulate proliferation of MCF7 cells as found previously for other breast cancer cell lines (6,8). However, the previous studies used WF in serum-free medium with peripheral blood serum as controls whereas MCF7 cells required 3% FBS for short-term and clonogenic proliferation. Furthermore, a significant reduction of WF-stimulated proliferation by IORT was not observed in short-term 2-D cultures of MDA-MB-231 (slight decrease;  $P=0.11$ ), MDA-MB-453 (slight increase;  $P=0.2$ ), or SKBR-3 (slight increase;  $P=0.1$ ) in the study by Belletti *et al.* (8). Notably, they did not show data for MCF7 in 2-D culture but the non-significant decrease ( $P=0.07-0.16$ ) observed for MCF7 in the present study together with the previous findings supports the conclusion that WF from IORT patients does not impair proliferation in 2-D culture. This was corroborated by the absence of an effect of IORT on WF-stimulated entry into the S-phase of the cell cycle (8).

It should be noted that the present results do not rule out that there may be differences in the cellular microenvironment and the cytokine composition of WF after IORT compared to no IORT. Thus, Belletti *et al.* (8) showed that IORT reduced WF-stimulated migration (chemotaxis assay) and invasion (Matrigel Transwell assay). This was associated with changes in the cytokine profile of WF by intraoperative tumor-bed irradiation as performed according to the TARGIT protocol (8). In addition, the size of MCF7 colonies grown in a Matrigel 3-D matrix was reduced. However, since 2-D area rather than cell numbers per colony or yield of colonies per cell seeded was measured, proliferation may have been confounded by invasion and migration in this assay.

In summary, the present study did not support an effect

of WF from IORT patients on clonogenic or short-term proliferation of MCF7 breast cancer cells.

### Acknowledgments

We would like to thank the staff of the Department of Radiation Oncology and the Department of Gynecology and Obstetrics (UMM, Mannheim) for their support.

*Funding:* The original study on IORT was funded by the German Federal Ministry of Education and Research (BMBF; grant FKZ01ZP0508).

### Footnote

*Provenance and Peer Review:* This article was commissioned by the editorial office, *Translational Cancer Research* for the series "Intraoperative Radiotherapy II". The article has undergone external peer review.

*Conflicts of Interest:* Radiobiological research in our department is supported by Carl Zeiss Surgery, Oberkochen, Germany. FG, MS and FW receive speaker's fees and honoraria by Carl Zeiss Meditec AG. For all other authors, there are no conflicts of interest.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Medical Ethics Commission II of the Medical Faculty of Mannheim, Heidelberg University and was conducted according to Declaration of Helsinki principles. Informed consent was obtained.

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**Cite this article as:** Veldwijk MR, Neumaier C, Gerhardt A, Giordano FA, Sütterlin M, Herskind C, Wenz F. Comparison of the proliferative and clonogenic growth capacity of wound fluid from breast cancer patients treated with and without intraoperative radiotherapy. *Transl Cancer Res* 2015;4(2):173-177. doi: 10.3978/j.issn.2218-676X.2015.04.01