

Targeted Intraoperative Radiotherapy Impairs the Stimulation of Breast Cancer Cell Proliferation and Invasion Caused by Surgical Wounding

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Abstract Purpose: After apparently successful excision of breast cancer, risk of local recurrence remains high mainly in the area surrounding the original tumor, indicating that wound healing processes may be implicated. The proportional reduction of this risk by radiotherapy does not depend on the extent of surgery, suggesting that radiotherapy, in addition to killing tumor cells, may influence the tumor microenvironment.

Experimental Design: We studied how normal and mammary carcinoma cell growth and motility are affected by surgical wound fluids (WF), collected over 24 h following breast-conserving surgery in 45 patients, 20 of whom had received additional TARGeted Intraoperative radiotherapy (TARGIT), immediately after the surgical excision. The proteomic profile of the WF and their effects on the activation of intracellular signal transduction pathways of breast cancer cells were also analyzed.

Results: WF stimulated proliferation, migration, and invasion of breast cancer cell lines. The stimulatory effect was almost completely abrogated when fluids from TARGIT-treated patients were used. These fluids displayed altered expression of several cytokines and failed to properly stimulate the activation of some intracellular signal transduction pathways, when compared with fluids harvested from untreated patients.

Conclusions: Delivery of TARGIT to the tumor bed alters the molecular composition and biological activity of surgical WF. This novel antitumoral effect could, at least partially, explain the very low recurrence rates found in a large pilot study using TARGIT. It also opens a novel avenue for identifying new molecular targets and testing novel therapeutic agents.

Breast-conserving surgery followed by external radiotherapy is the current standard treatment for localized cancer of the breast (1, 2). Conventional wisdom is that radiotherapy sterilizes the tumor bed of residual tumor cells. However, the absolute effect

of radiotherapy in reducing the risk of local recurrence increases with increasing risk of local recurrence (e.g., with less extensive surgery). Thus, the *proportional* reduction of the risk by radiotherapy (to about one third of the background risk) remains constant (3). Despite the presence of multiple foci of cancer in about two thirds of mastectomy specimens (4–6), recurrences localize most frequently within the area surrounding the original tumor. Moreover, although the total risk for a woman to develop a breast cancer in her life is only 8%, ~33% of middle-aged women have undiagnosed breast cancer, as evidenced by medico-legal autopsies (7). All these clinical observations, coupled with the notion that surgery modifies the growth kinetics of breast cancer micrometastasis (8, 9), support the hypothesis that surgery itself represents a perturbing factor for metastasis development in humans, as shown in animal models (10, 11). Accordingly, axillary wound fluid (WF) derived from breast cancer patients induced proliferation of Her2/Neu-expressing breast carcinoma cells *in vitro*, an effect that could be abrogated by trastuzumab (12). Thus, it is possible that modification of the local microenvironment by surgery may alter the growth kinetics of cancer cells, supporting the “seed and soil” theory first proposed by Sir Stephen Paget to explain the pathogenesis of cancer metastasis (13, 14). Interestingly, it has been hypothesized that radiotherapy may affect not only breast cancer cell survival but also the tumor

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Received 9/26/07; revised 11/23/07; accepted 12/1/07.

Grant support: Associazione Italiana per la Ricerca sul Cancro and Ministero della Salute (G. Baldassarre); Alleanza Contro il Cancro, project Proteomics (A. Colombatti); and fellowship from Associazione Italiana per la Ricerca sul Cancro (F. Lovat).

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doi:10.1158/1078-0432.CCR-07-4453

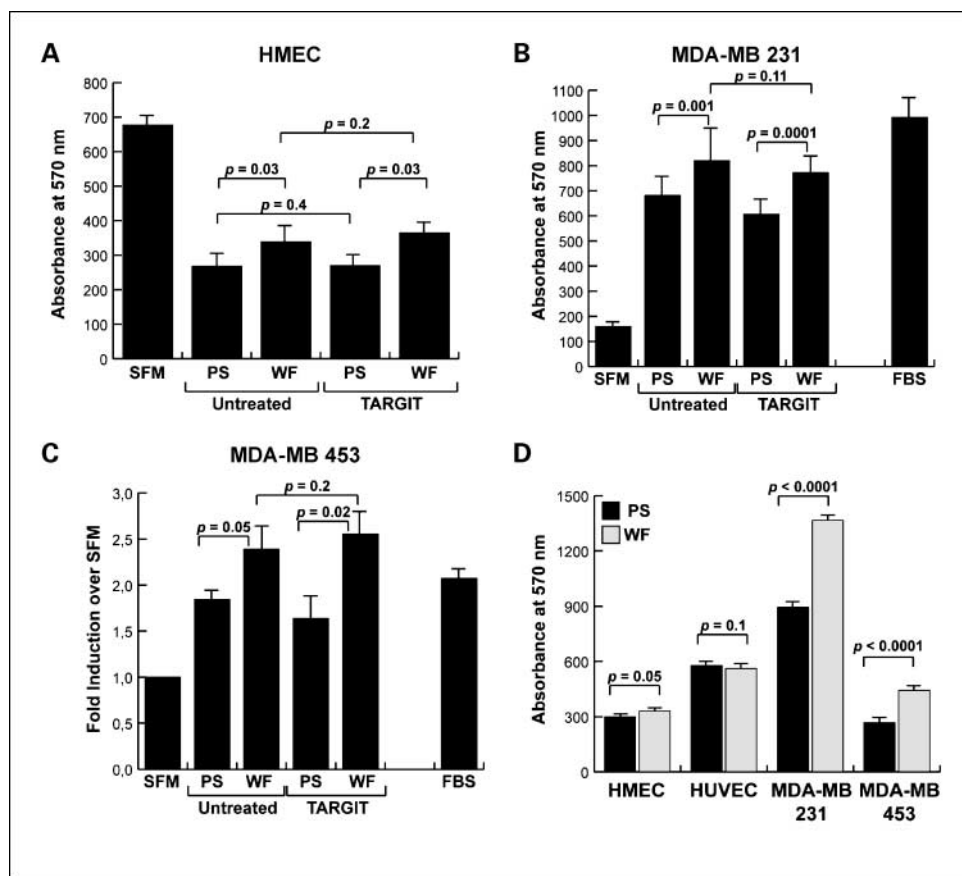


Fig. 1. WF stimulate cancer cell proliferation in two dimensions. HMEC (A) and mammary carcinoma derived cell lines MDA-MB 231 (B) and MDA-MB 453 (C) were incubated for 4 d in the presence of the indicated sera at 1% in serum-free medium (SFM). A, SFM represents 30% of HMEC-specific medium and PS and WF at 1% were added to this medium. B and C, SFM is 0.1% bovine serum albumin in DMEM and addition of 10% fetal bovine serum (FBS) was used as positive control. Cells were then labeled with MTT reagent. Viable cells were evaluated by reading the absorbance at 570 nm. Columns, mean from cells treated with 20 different PS and WF samples for each group of patients (each serum has been evaluated in sextuplicate); bars, SD. D, sera from all preoperative drawings (PS) and WF from 20 TARGIT-treated and 25 untreated patients were pooled and the relative mixes were assayed for cell proliferation by MTT assay. Pools of WF from TARGIT-treated and untreated patients provided similar results, which have therefore been combined in the graph (gray columns). Columns, mean of two independent experiments done in sextuplicate; bars, SD. Statistical analyses have been done using the Student *t* test.

microenvironment by changing the cell phenotype, the tissue composition, and the physical interactions and signaling between cells (15, 16).

The preponderance of local recurrence along the scar produced by the surgical excision has stimulated clinical trials to test if localized radiotherapy, using intraoperative (17, 18), interstitial, or three-dimensional conformal techniques (19, 20), would be preferable to conventional whole-breast radiotherapy. Targeted intraoperative radiotherapy (TARGIT) is the first of such techniques and enables delivery of radiotherapy to the tumor bed immediately after surgical excision of the tumor. Given in place of a tumor bed boost, TARGIT achieves a lower than expected recurrence rate (21). Its efficacy as single radiotherapy treatment is being tested in an international randomized trial. The TARGIT technique offers a unique opportunity to study the immediate effect of radiotherapy on human tissues *in vivo*.

We investigated whether TARGIT influenced the responses of breast cancer cell lines after stimulation with surgical WF, in terms of cell proliferation, motility, and invasion. We also carried out preliminary proteomic analyses of the fluids to assess which factors may be responsible for the observed effects. By these experimental approaches, we tried to answer two clinical/biological questions: First, why do local recurrences arise mainly in the tumor bed irrespective of the margin status? Second, does intraoperative radiotherapy have an immediate effect on the local tumor microenvironment? Is it therefore conceivable that in addition to killing residual tumor cells ("the seed"), radiotherapy also alters the micro-

environment ("the soil"), making it less favorable for tumor cell growth and invasion in humans, as already shown in animal models (22)?

Materials and Methods

Patients and sample collection. Preoperative peripheral blood serum (PS) and drainage WF over the first 24 h after surgery were collected from each of 45 unselected patients (Supplementary Table S1) undergoing breast-conserving surgery. Twenty of these patients were treated with TARGIT (refs. 16, 17; TARGIT, 20 Gy to tumor bed surface in one session) immediately after the surgical excision. The WF was collected with the usual surgical suction drain. The perforated end of the surgical drain is placed in the wound; the tubing exits the wound through a separate tight hole and the other end is placed in an evacuated sterile container. The drainage tube is removed at 24 h after surgery. Sera and WF collected over 24 h were centrifuged, sterile filtered, and stored at -80°C . This study was approved by the Ethics Committee of the Centro di Riferimento Oncologico, Aviano.

We grouped patients into those who had received TARGIT and those who had not. Each patient had two samples: PS and 24-h WF. Some assays were done using a pool of all fluids taken from each group, whereas others using individual patient samples. This is specified under the description of the individual experiments.

Cell lines and treatments. Experiments were done on the following breast cancer cell lines: MCF-7 and T47D cells (ER/PgR+; Her2/Neu-), MDA-MB-231 (ER/PgR-; Her2/Neu-), and MDA-MB-453 and SKBR-3 (ER/PgR-; Her2/Neu+). Normal human mammary epithelial cells (HMEC; Clonetics), human umbilical endothelial cells (isolated in our laboratory following published procedures; ref. 23), and normal

mouse mammary gland epithelial cells (NMuMG; kind gift of Dr. Andrej Bakin, Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY) were used as controls.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, fluorescence-activated cell sorting analysis of DNA content, and cell growth in three-dimensional matrices, such as collagen I or Matrigel, were used to evaluate proliferation. Motility was studied using chemotaxis, standard Matrigel invasion assay, and video time-lapse microscopy of cells included in three-dimensional collagen I. These assays were done essentially as previously reported (24) and a detailed description is included in Supplementary data.

Proteomic analysis. To investigate the molecular changes in the WF following TARGIT, we used three pools of samples: PS (PS from both TARGIT-treated and untreated patients), WF from untreated patients, and WF from TARGIT-treated patients. These samples were then assayed using the RayBio Human Cytokine Antibody Array C that detects 174 cytokines (RayBiotech) following the manufacturer's instructions. Expression of each cytokine was calculated by densitometric analysis of the blots using a Bio-Rad Gel Doc 2000 apparatus interfaced with Quantity One software. For each membrane, cytokine expression was normalized using the positive and negative controls present on the array as suggested by the manufacturer's instructions. Only those quantitative changes present in two separate experiments were considered specific. ELISA kits specific for human interleukin (IL)-1 β , IL-5, IL-10, IL-13, hepatocyte growth factor (HGF), and leptin (Biosource) were used on single WF to validate the proteomic results.

Phosphoproteomic analysis. MCF-7, T47D, and MDA-MB-231 cells were serum starved overnight in DMEM-0.1% bovine serum albumin and then stimulated with 2.5% of pool of PS, WF, or WF from TARGIT-treated patients for 5, 15, 30, and 60 min. Cells were then lysed and analyzed by Western blot with commercially available antibodies directed against known activating phosphoepitopes (see Supplementary data for a complete list).

Statistical methods. Data were examined using the two-tailed Student *t* test except for the analysis of cell speed and motility for which the more appropriate unpaired two-tailed Mann-Whitney *U* test was used. Differences were considered significant at *P* < 0.05. The computer program PRISM (version 4, GraphPad, Inc.) was used.

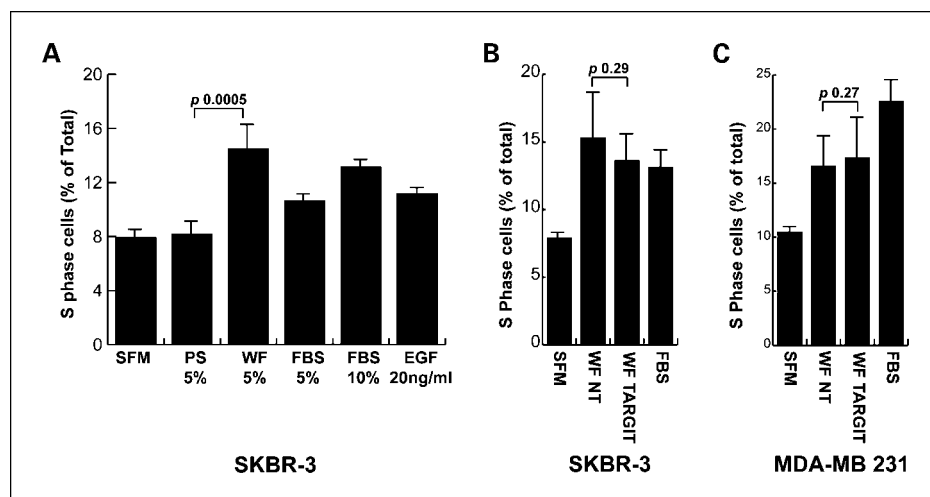
Results

Effects of WF on tumor and normal cell proliferation in two dimensions. First, we verified whether we could confirm previous observations on the ability of wound axillary fluids to stimulate mammary cancer cell proliferation (12) by testing

the proliferation of normal or tumor-derived cell lines following stimulation with WF. We carried out MTT proliferation assay using individual PS and WF from each patient, as shown in Figure S1A and B, on MDA MB 231 and NMuMG cells. The same experiment with individual sera was also done on HMEC, MDA MB 453, and SKBR-3 cells (Fig. 1A-C and Supplementary Fig. S1C and D), but in these cases the results are reported as the average value of absorbance for each category of sera. We observed that the stimulation of the growth of mammary carcinoma cell lines by WF was significantly higher than that produced by the respective PS (Fig. 1 and Supplementary Fig. S1). This effect was observed for WF from all patients, including those who had received TARGIT (Fig. 1 and Supplementary Fig. S1). The MTT assay is a high-throughput test that quite easily allows the simultaneous evaluation of many samples on even multiple cell lines. Yet, to be able to carry out other kinds of assays, which could more precisely indicate biological activity and mechanisms of action of the sera, we needed to reduce the complexity of our model system. To this aim, we prepared four pools of sera: (a) PS from the group of untreated patients, PS untreated; (b) WF from the group of untreated patients, WF untreated; (c) PS from the group of TARGIT-treated patients, PS TARGIT; (d) WF from the group of TARGIT-treated patients, WF TARGIT. Then, we assessed the biological validity of these pools by repeating the MTT assay on some of the cell lines already challenged with the individual sera. Data reported in Fig. 1D show the reliability of the results obtained with pools because they do not significantly differ from those obtained with individual sera, and thus allow us the confident use of these pools in some of the experiments reported herein.

Some individual sera (*n* = 6 from women that received TARGIT and 6 from untreated patients) were also evaluated for their ability to induce cell cycle reentry after starvation by fluorescence-activated cell sorting analysis using SKBR-3 and MDA-MB 231 cells. WF stimulated S-phase entry of the cells significantly better than respective PS (Fig. 2A and data not shown). Interestingly, WF at 5% induced cell cycle entry of SKBR-3 Her2/Neu+ cells significantly better than 20 ng/mL epidermal growth factor. Again, TARGIT treatment did not significantly modify the ability of WF to induce S-phase entry of these cells (Fig. 2). Normal HMEC need specific medium

Fig. 2. WF stimulate the S-phase entry of mammary carcinoma cells. **A**, percentage of S-phase population of SKBR-3 mammary carcinoma cells, serum starved for 24 h (SFM) and then stimulated for 12 h with the indicated amount of PS or WF. Fetal bovine serum at 5% and 10% and epidermal growth factor (EGF) at 20 ng/mL were used as positive controls. Columns, mean of two independent experiments in which six randomly selected WF from untreated and six from TARGIT-treated patients and their respective PS were tested; bars, SD. **B** and **C**, percentage of S-phase population of SKBR-3 (**B**) or MDA-MB-231 (**C**) mammary carcinoma cells, serum starved for 24 h (SFM) and then stimulated for 12 h with 5% WF from untreated or TARGIT-treated patients or with 10% fetal bovine serum used as positive control. Columns, mean of two independent experiments in which six randomly selected WF from untreated and six from TARGIT-treated patients were tested; bars, SD. Statistical analyses have been done using the Student *t* test.



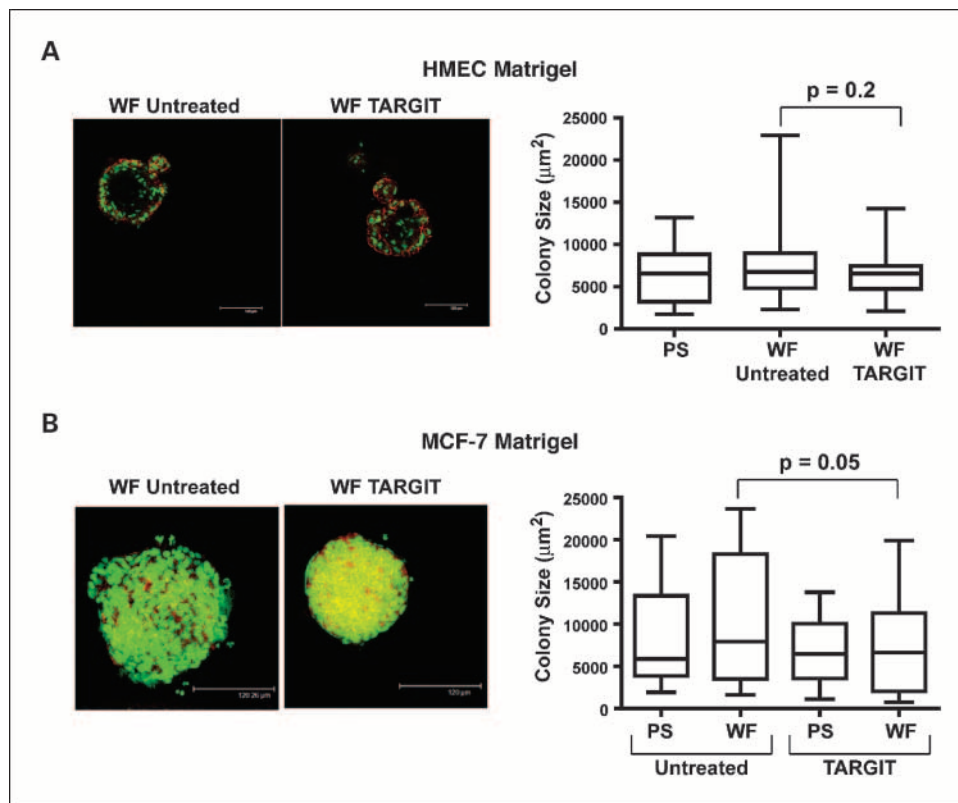


Fig. 3. TARGIT treatment impairs the WF-induced cancer cell growth in three-dimensional matrices. HMEC (A) and MCF-7 cells (B) were included in a thick three-dimensional Matrigel layer and allowed to grow for 15 d. Cells were then stained with phalloidin (red) and Cytochrome green (green) to visualize the actin cytoskeleton and the nuclei. Analysis by confocal microscopy was carried out by performing sections every 10 μm . The maximum colony area was evaluated for at least 100 colonies and reported in the graphs (C and D). Area of the colonies was measured using Leica LAS software and data are reported in the box plots. The median value (line within the box), the interquartile range representing 50% of the data (boundaries of the box), and the spread (vertical lines) representing the highest and lowest value (horizontal lines) are shown. Statistical analyses have been done using the Student *t* test.

supplemented with numerous growth factors to grow *in vitro*. WF were unable to stimulate HMEC growth at the level of their specific medium, yet they stimulated HMEC proliferation at higher levels with respect to PS (Fig. 1A). On the contrary, the growth of normal mouse mammary cells stabilized in culture (NMuMG) was stimulated more by PS than by WF (Supplementary Fig. S1B and D) whereas normal human umbilical vein endothelial cells were equally stimulated by PS and WF (Fig. 1D and data not shown). These results show that the effects of WF on normal mammary cell growth are mild and variable, whereas those on mammary carcinoma cells are constantly stimulatory. In all cases, no significant difference was observed in the ability to stimulate cell growth by WF derived from untreated or TARGIT-treated patients.

Effects of WF on breast cancer cell growth in a three-dimensional model. It is largely accepted that cells proliferate differently when cultured in two-dimensional with respect to three-dimensional conditions (25), and recent data coupled with mathematical models also showed how the stiffness of the substrate could influence the growth of mammary carcinoma cells (26, 27). HMEC and three different breast cancer cell lines (MDA-MB 231, T47D, and MCF-7) were assayed for their ability to grow in a three-dimensional matrix using both collagen I and Matrigel, following a well-validated protocol (28). When grown in three-dimensional Matrigel or collagen I, HMEC cultured in the presence of pools of PS, WF, or WF from TARGIT-treated patients formed tubular structures that resemble the ducts of the mammary gland (Fig. 3A), as reported for normal cells grown in their culture medium (data not shown; ref. 28). Thus, in a three-dimensional context, WF and WF from TARGIT patients do not alter the ability of normal human

mammary cells to form tube-like structures, nor did the WF have a stimulatory effect over the control (i.e., the PS). However, when cancer cell lines were assayed for their ability to grow in three-dimensional Matrigel or collagen I, results were rather different. Both MCF-7 and T47D cells formed much larger colonies when cultured in the presence of WF compared with PS. Interestingly, this effect was abrogated when the WF was harvested from TARGIT-treated patients (Fig. 3B and Supplementary Fig. S2). Similar results were obtained with the MDA-MB 231 grown in three-dimensional Matrigel (Supplementary Fig. S2C).

Effects of WF on breast normal and cancer cell motility. Next, we assayed five cancer cell lines and two normal primary cultured cells in a transwell-based chemotactic assay, using individual patient fluids as potential chemoattractant (Supplementary Fig. S3C and D and data not shown) or the pools of sera (Fig. 4 and Supplementary Fig. S3A and B). WF strongly attracted cells from all five types of breast cancer cell lines tested, as shown for MDA-MB 231 (Fig. 2 and Supplementary Fig. S3C and D), MCF-7 (Supplementary Fig. S3A), MDA-MB 453 (Supplementary Fig. S3B), T47D, and SKBR-3 (data not shown). The WF attracted the cells to a larger extent than both the PS and the positive control (the conditioned medium from NIH 3T3 fibroblasts). More importantly, in all experiments and in all tested cell lines, TARGIT significantly impaired the ability of WF to attract cancer cells (Fig. 4 and Supplementary Fig. S3). On the contrary, the two types of WF stimulated migration of two normal cell lines, HMEC (Fig. 4A) and human umbilical endothelial cells (data not shown), to similar extent, suggesting that, also *in vivo*, normal and cancer cells could respond differently to wound healing after surgery.

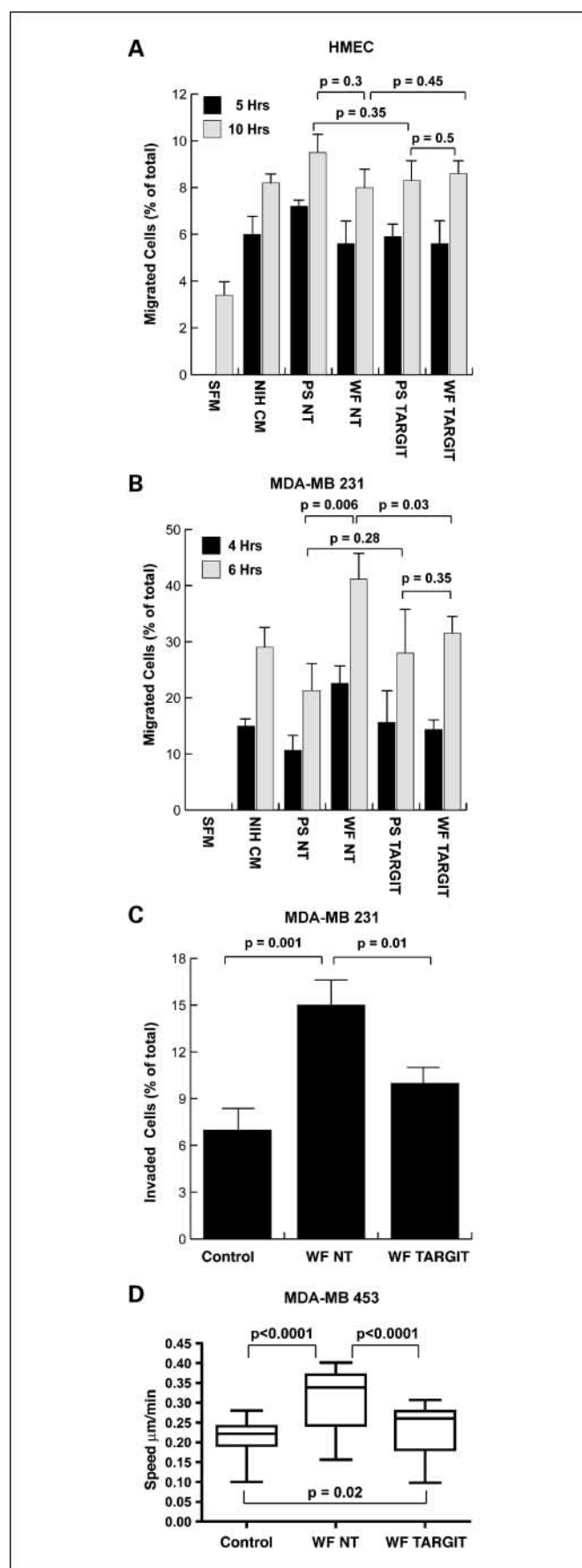
Effects of WF on breast cancer cell invasion. MDA-MB 231 cells were assayed for their ability to invade a three-dimensional Matrigel gel using a transwell-based assay. The pool of PS did not stimulate cell invasion over the control (Supplementary Fig. S4B). On the contrary, the use of the pool of WF (Fig. 4C) or of the single WF from individual patients ($n = 12$; Supplementary Fig. S4A and B) strongly stimulated cell invasion. Importantly, the stimulatory effect was abrogated when the WF came from patients who had received TARGIT (Fig. 4C).

Next, using video-time lapse microscopy, we assayed the ability of MDA-MB 453 to move within a three-dimensional collagen I gel in response to WF stimulation, using a previously validated assay (29). In this case, only the pooled samples were used. We found that WF increased both the single-cell speed (Fig. 4D) and the number of moving cells (Supplementary Fig. S4C), and again, this effect was abrogated when WF from TARGIT-treated patients were used. Thus, these data confirmed in a three-dimensional model (Fig. 4C and D) the results of the chemotaxis (Fig. 4B), supporting the hypothesis that factors released during wound healing process could also stimulate cancer cell motility *in vivo*.

Proteomic analysis of the WF. The different biological effects of PS, WF, and WF from TARGIT-treated patients could depend on changes in the proteomic profile of the different sera. To verify this hypothesis, growth factors and cytokines present in the different sera have been qualitatively and quantitatively evaluated (Fig. 5A). Interestingly, the pool of WF harvested from TARGIT-treated patients showed reduced expression of several molecules linked to tumor growth and motility, such as HGF (30), leptin (31), RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted; ref. 32), and uPAR (Urokinase-Type Plasminogen Activator Receptor; ref. 33; Table 1). To verify whether the differences observed in the WF pools reliably reflected the quantitative presence of the factors in each single WF, we used commercially available ELISA kits directed against some of the differentially expressed proteins. Quantitative analysis of HGF, IL-10, IL-13, IL-5, IL-1 β , and, to a lesser extent, leptin (Fig. 5B and data not shown) into single sera confirmed the semiquantitative results from the array since the expression level of each cytokine in the pool was representative of the median level of the same factor in the individual sera. These data may explain the different biological activities observed in proliferation and motility assays.

WF from TARGIT-treated patients fail to activate properly signal transducer and activator of transcription-3 and p70^{S6} kinase. Next, we carried out a phosphoproteomic screening to verify whether and how the different proteomic profiles of WF

Fig. 4. TARGIT treatment impairs the WF-induced cancer cell migration and invasion. HMEC (A) and mammary carcinoma – derived cell line MDA-MB 231 (B) were tested in a transwell-based chemotaxis assay for their ability to migrate toward the indicated treatment. PS and WF pools were used at 2.5% in SFM. Conditioned medium from NIH 3T3 fibroblasts was used as positive control. Columns, mean percentage of migrated cells at two different time points from three independent experiments done in duplicate; bars, SD. C, percentage of MDA-MB 231 cells invading a three-dimensional Matrigel gel in a transwell-based chemotaxis assay in response to the indicated WF, used at a concentration of 2.5%. Control is represented by the medium supplemented with 10% fetal bovine serum. Columns, mean of two independent experiments done in duplicate; bars, SD. D, single-cell speed of MDA-MB 453 immersed in a three-dimensional collagen I matrix and treated as indicated. Forty cells per treatment were followed by a cell tracking software in each experiment and data represent the mean of three experiments. Statistical analyses were done using the Student *t* test (A-C) and the Mann-Whitney *U* test (D).



and PS could affect the activation of intracellular pathways in breast cancer cells. Among the several intracellular signal transduction pathways analyzed in MCF-7, T47D, and MDA-MB-231 (Supplementary Figs. S5 and S6 and data not shown), the activation of the signal transducer and activator of transcription-3 (STAT3) and p70^{S6} kinase pathways significantly differed when the cells were stimulated with the pool of PS, control WF, or WF from TARGIT-treated patients. In fact, control WF induced a strong and prolonged phosphorylation of STAT3 on Y705 (but not on S727) in both MCF-7 (Fig. 4C, top) and T47D (Fig. 4D, top). Yet, when cells were treated with PS or WF from TARGIT-treated patients, Y705 phosphorylation was significantly lower (Fig. 4C and D, top). Similarly, stimulation with WF strongly activated p70^{S6} kinase, as revealed by its phosphorylation on T389 and by the phosphorylation of its substrate S6 ribosomal protein at S235/236 and S240/244 (Fig. 5C and D, 3rd to 5th rows). Yet, pools of PS or WF from TARGIT-treated patients were not able to achieve comparable activation levels. Similar results were obtained using MDA-MB-231 cells (data not shown).

Thus, the differences in the proteomic profile between WF from untreated and those from TARGIT-treated patients concretely resulted into an impaired activation of the STAT3 and p70^{S6} kinase signaling pathways, suggesting a molecular explanation for the different biological activities exerted by these sera.

Discussion

The preferential localization of breast cancer recurrences at the surgical scar site has not yet been adequately explained, and various hypotheses have been hitherto put forth. The most common explanation is the presence of "residual" tumor cells. However, this cannot be the only explanation.

Although residual tumor cells may still be present even when meticulous care is taken to ensure that excision margins are microscopically free of tumor, it is difficult to explain why radiotherapy reduces the risk of recurrence by the same proportional extent however wide the excision is. The possible deleterious effects of surgical wounding have been speculated for a long time and have been shown in mice (8, 10, 11). Moreover, experimental and clinical observations suggest that the extent of surgery may represent a variable able to enhance tumor burden (11, 36). This effect has previously been related to growth factor produced by stromal cells during the wound healing process (12, 37). Interestingly, wound axillary fluids harvested from breast cancer patients have been proved to stimulate Her2-positive mammary carcinoma cell growth in two-dimensional assay, an effect that could be only partially abrogated by impairing Her2 signal transduction (12). This observation implies that several growth factors and cytokines secreted in the WF participate in the stimulation of mammary carcinoma cells as our biological and biochemical data suggest. Our work not only confirms the previous work of Tagliabue et al. (12) but also represents the first formal demonstration that WF harvested from breast cancer patients who have undergone wide local tumor excision stimulate breast cancer cell motility, invasion, and growth in three-dimensional contexts. Thus, the WF may stimulate the growth of any residual tumor cell and/or attract breast cancer cells at the site of surgery, suggesting an additional molecular and biological explanation for the high local recurrence rate of breast cancer.

Irrespective of the stage of the tumor, the hazard of local as well as distant relapses peaks between 2 and 3 years after primary surgery (9). Improving local control improves overall survival (3), albeit not at the same proportion. This suggests that events around the primary surgical operation may be crucial in determining ultimate prognosis (9). Surgical excision of the

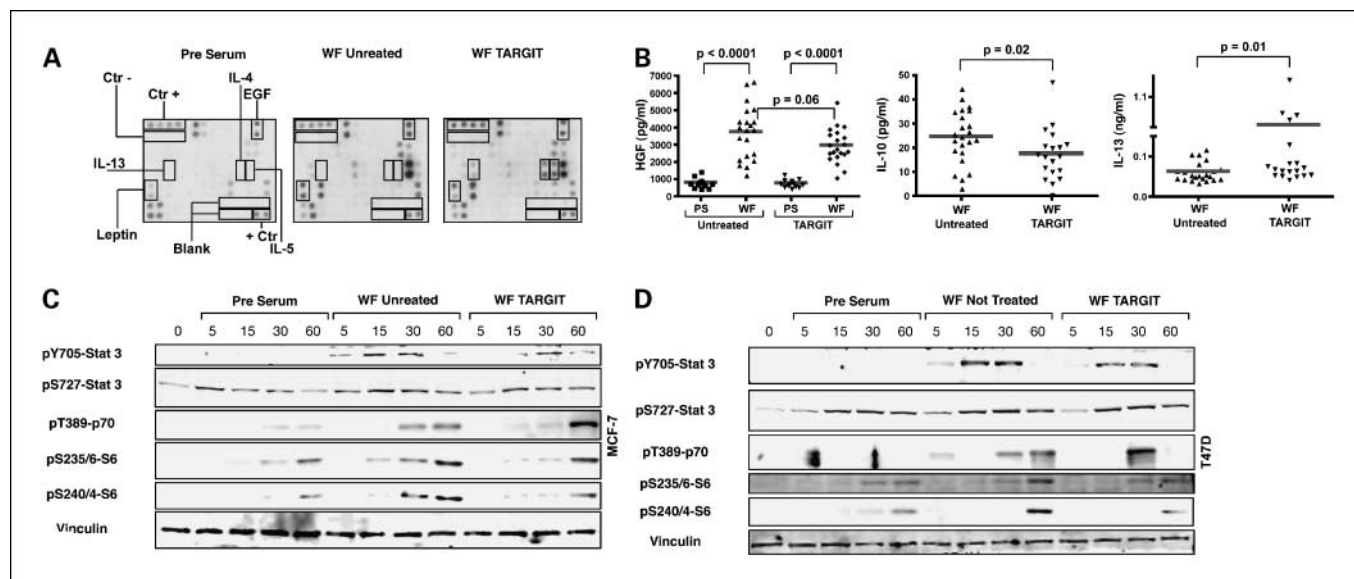


Fig. 5. TARGIT alters the proteomic profile of WF and the activation of p70^{S6} kinase and STAT3 pathways on stimulation of breast cancer cells. **A**, typical image of membranes spotted with different growth factors and hybridized with pools of PS, WF from untreated patients, and WF from TARGIT-treated patients, as indicated. The position of spots containing positive (Ctr+) and negative (Ctr-) controls, blank, and some differentially expressed proteins are highlighted in boxes. **B**, quantification of leptin, HGF, IL-10, and IL-13 expression in the WF and PS of each patient by ELISA. IL-10 and IL-13 expression were not detectable in PS sample and, thus, not included in the graph. **C** and **D**, Western blot analysis of the expression of the indicated phosphoproteins in MCF-7 (**C**) and T47D (**D**) cells. Cells were serum starved (lane 0) and then stimulated with the indicated sera at 5% in SFM for 5, 15, 30, and 60 min. Vinculin expression was used as loading control.

Table 1. WF proteins altered by TARGIT

Decreased by TARGIT	Increased by TARGIT
Angiogenin	AgRP
Flt-3 ligand	EGFR
IL-10	FAS/TNFRSF6
IL-6	FGF-4
IL-7	G-CSF
Leptin	IGFBP-6
MCP-1	IL-13
MCP-2	IL-4
RANTES	IL-5
PDGF-BB	Mip-1d
GRO	
HGF	
IL-8	
Mip-1a	
sTNFR-II	
sTNFR-I	
uPAR	
VEGF-R3	
Tie-1	
Tie-2	

NOTE: Only those proteins (of 174 analyzed) that significantly differed between WF from untreated and from TARGIT-treated patients are listed in the table. Semiquantitative analysis was done in duplicate in two separate experiments.

Abbreviations: AgRP, agouti-related protein; EGFR, epidermal growth factor receptor; FGF-4, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GRO, growth-regulated oncogene; MCP-1 and MCP-2, monocyte chemotactic proteins 1 and 2; Mip-1a and Mip-1d, macrophage inflammatory proteins 1a and 1d, respectively; PDGF-BB, platelet-derived growth factor BB; sTNFR-I and sTNFR-II, soluble tumor necrosis factor receptor type I and type II, respectively; Tie-1 and Tie-2, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 1 and 2, respectively; TNFRSF6, tumor necrosis factor receptor superfamily member 6; VEGF-R3, vascular endothelial growth factor receptor 3.

cancer is certainly beneficial but the act of surgery may have harmful effects (8, 9) and we should strive to understand and reduce them. We show here that TARGIT almost completely abrogates the stimulatory effects of surgical WF on cancer cells *in vitro*, suggesting that it may confer more benefits than those expected from the tumoricidal effect of radiotherapy. Our observations are in line with the clinical evidence. It has been shown that tumor bed boost delivered with conventional external beam radiotherapy significantly improves local control

(38). Notably, TARGIT used as a tumor bed boost in 301 unselected breast cancer patients resulted in a better local control if compared with the one obtained with the external beam radiotherapy boost (1.9% actuarial at 5 years compared with the expected 5-8%), with only one recurrence (a focus of ductal carcinoma *in situ*) occurring at the site of the primary tumor (21). Perhaps, the beneficial effect of TARGIT could have contributed to achieving such a low rate of recurrence not only through a better cell killing (which is probably the main mechanism of action) but also by modifying the wound microenvironment, making it less favorable for cancer cell growth and invasion. Of course, this study does not provide a proof for the superiority of intraoperative radiotherapy, but it does however provide a biological rationale for that possibility.

Although our proteomic analysis is partial because it is based on the evaluation of known cytokines, it is at least partially coincident with similar analysis done in mouse models in which, for example, HGF and uPA production have been identified in sera 24 h after surgery (11). Little is known about the effects of radiotherapy on cytokine expression modification in humans after surgery; however, specific increase in IL-5 and IL-4 following radiation therapy has been observed both in human and rats (39-41), supporting our results that TARGIT significantly modifies the protein expression profile of the WF. Interestingly, many of the factors and pathways identified as "modified by TARGIT" in our proteomic analysis (e.g., IL-6, RANTES, HGF, or leptin and the STAT3 and p70^{S6} kinase) have been already implicated in the control of cancer cell growth and motility and considered as targets of new anticancer therapies (30-35, 42).

It is also important to note that in mice the beneficial effects of radiotherapy on the tumor bed are highly dependent on the time at which cancer cells are implanted in the irradiated tissue (22), suggesting that administration of radiotherapy immediately after surgery could represent a more effective measure to reduce local recurrence of cancer.

In conclusion, this study provides a proof of principle and a biological rationale for the idea that perioperative treatments, such as TARGIT, could reduce tumor recurrence by beneficially altering the tumor microenvironment, in addition to the conventionally known tumoricidal effect. These findings may also stimulate research into the development of novel perioperative local and systemic treatments especially directed to compensating the harmful consequences of surgery and wound healing.

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